

OLFACTORY DISCRIMINATION OF EWE  
URINE ODOURS BY RAMS.

Martyn Blissitt.

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I dedicate this work to everyone  
who likes sheep.

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I learnt much from four individuals, who must remain nameless, the rest of this work is mine.

A handwritten signature in dark ink, consisting of a series of loops and a long horizontal stroke at the end.

FOREWORD.

They haven't got no noses,  
The fallen sons of Eve;  
Even the smell of roses  
Is not what they supposes;  
But more than mind discloses  
And more than men believe.

The brilliant smell of water,  
The brave smell of a stone,  
The smell of dew and thunder,  
The old bones buried under,  
Are things in which they blunder  
And err if left alone.

The wind from winter forests,  
The scent of scentless flowers,  
The breath of brides' adorning,  
The smell of snare and warning,  
The smell of Sunday morning,  
God gave to us for ours.

And Quoodle here discloses  
All things that Quoodle can,  
They haven't got no noses,  
They haven't got no noses,  
And goodness only knows  
The Noselessness of man.

G.K.Chesterton.



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### SUMMARY.

Four sexually experienced adult rams were trained to use an operant conditioning apparatus which enabled them to perform simultaneous olfactory discrimination experiments.

#### Frozen/Thawed urine samples.

1. Rams could discriminate between the odours of defrosted oestrous (day 1) and non-oestrous (day 9) ewe urine, collected from the same ewe, when the urine had been frozen for periods ranging from 30 minutes to 250 days. The duration of freezing did not appear to affect the rams ability to discriminate between urine odours.
2. The odour of fresh non-oestrous urine was aversive to rams when initially presented, but the aversion was quickly overcome and could not be demonstrated subsequently.
3. Ram's abilities to discriminate between defrosted, oestrous and non-oestrous urine odours was transferable to trials with previously untested urine odours from different oestrous cycles and other ewes.
4. The processes of freezing and thawing changed the odours of both oestrous and non-oestrous ewe urine as perceived by rams. Defrosted ewe urine odours are therefore artifactual. This severely limits the usefulness of conclusions that can be drawn from experiments using defrosted ewe urine samples.

### Fresh urine samples.

Twenty two olfactory discrimination experiments were conducted using fresh oestrous and non-oestrous urine samples from different ewes and different oestrous cycles. The only consistent feature of the urine samples in each discrimination experiment was that one was from a sexually receptive ewe whilst the other was from a sexually unreceptive ewe. Rams could discriminate between the odours in every experiment. During the first 12 experiments the rams learnt to respond to the consistent difference in odour between the two types of urine. In the last 10 experiments, rams were able to choose the new oestrous urine sample consistently in the initial trial. These results indicate that rams are able to differentiate between sexually receptive and non-receptive ewes on the basis of their urine odour.

### Location of Mediating chemoreceptors.

1. Vinyl polysiloxane bungs were used to occlude the nasopalatine canals (NPC) and so block chemical access to the vomeronasal organ (VNO). Irrigation of the nose with zinc sulphate was used to induce olfactory deficits. The performances of rams after such treatments were assessed in 3 ways:

- a) Oestrous v. non-oestrous urine odour discriminations.
- b) Determining the ability of rams to flehmen.
- c) Determining the ability of rams to perceive aversive odours.

2. NPC occluded rams discriminated between oestrous and non-oestrous urine odours, performed flehmen, and detected aversive odours.

3. One zinc sulphate treated ram showed olfactory deficits in all tests and did not flehmen (deficit >21 days). The other zinc sulphate treated ram detected aversive odours but did not discriminate between urine odours, and did not perform flehmen for the first 7 days post-treatment. After 7 days the ram showed normal olfactory abilities in all tests. Zinc sulphate applied in this way may temporarily destroy some chemoreceptive fields and not others, selectively maintaining some olfactory function.

4. It is concluded that in adult rams the VNO is not necessary for discrimination between oestrous and non-oestrous ewe urine odours, or for the flehmen response. The evidence suggests that chemoreceptors mediating this discrimination are located intranasally in the main olfactory epithelium.

## GENERAL INTRODUCTION.

This thesis concerns an investigation into the ability of rams to detect odours in ewe urine which indicate the ewe's sexual receptivity.

Rams are believed to use olfaction to detect oestrous ewes at a distance (Lindsay 1965) and behavioural observations indicate that ewe urine is a source of chemical information which rams use to predict oestrus (Geist 1971; Bland & Jubilan 1987). After olfactory bulbectomy (which eliminates sensory input from the main and accessory olfactory systems) rams mate with fewer ewes than intact rams because they are deficient at detecting oestrus (Fletcher & Lindsay 1968). Anosmia does not otherwise affect the mating ability of rams.

The flehmen response is commonly believed to facilitate the entry of chemical information, or pheromones, into the vomeronasal organ. The vomeronasal organ is believed to be the location of chemoreceptors which mediate the detection of these pheromones. However the identity, source and nature of putative pheromones; and the role of flehmen and the vomeronasal organ, has not been determined.

An understanding of these problems is important to improve our knowledge of odour communication and oestrous detection in domestic ruminants. Identification of oestrous-indicating pheromones would facilitate the development of new methods for detecting oestrus.



## CHAPTER 1. REVIEW OF LITERATURE.

### INTRODUCTION.

The domestication of wild animals has been termed the single most important intervention man has ever made in his environment (Isaac 1970). The sheep was the first of the present day farm animals to be domesticated, probably by mesolithic peoples in South-West Asia around 9000 B.C (Hilzheimer 1936; Ryder 1983). Of the wild types of sheep, the Urial (Ovis orientalis - Lydekker 1912) is thought to have been domesticated first. Two other wild types, the Moufflon (Ovis musimon) and the Argali (Ovis ammon) contributed to European and Asiatic breeds of the present day. In addition, Geist (1971) recognises three more wild types: Ovis canadensis - the Bighorn; Ovis nivicola - the snow sheep of Siberia; and Ovis dalli - the Thinhorn of Alaska. There is no evidence that these three types have ever been domesticated. The recognition of primitive types of sheep is important for the study of sheep behaviour. The behaviour of animals in their natural environment can be compared with the behaviour of domestic types subjected to modern agricultural methods.

### REPRODUCTION.

#### Breeding season.

Ewes are seasonally polyoestrous. The annual breeding season from Autumn to Spring is induced by shortening daylength (Marshall 1937; Yeates 1949).

Within this season oestrus recurs, in the absence of the male, every 16-17 days (range 16-18 days Hafez 1952) but varies slightly according to breed (Asdell 1964) and age (Hafez 1952; Lambourne 1956). Oestrus generally lasts 24-48 hours (Goodman 1988) but varies considerably. Breeds which shed 3-4 ova at ovulation have oestrous periods 50% longer than breeds shedding 1-2 ova (Land 1970; Quirke, Hanrahan & Gosling 1979). The duration of oestrus is longest in the middle of the breeding season (Fletcher & Lindsay 1971). Social interaction with rams can affect the duration of oestrus. If rams are given continuous access to an oestrous ewe then oestrus is half as long as that when access is allowed every four hours (Parsons & Hunter 1967; Van de Westhuyen, Van Neikirk, Hunter 1970). The continuous presence of rams shortens the interval between the start of oestrus and the luteinising hormone (LH) surge. The interval between the LH surge and ovulation is fixed, therefore the presence of rams reduces the duration of oestrus before ovulation occurs (Lindsay et al. 1975; Signoret 1975b). In the absence of rams ovulation can be delayed up to 12 hours, which may allow the ewe a greater opportunity to find a ram.

Most rams are capable of breeding all year round, but there are declines in parameters of sexual behaviour (such as latency to serve and number of services per unit time) which coincide with increasing daylength and low testosterone levels (Lincoln & Short 1980; Bremner,



Cumming, Winfield, de Kretster & Galloway (1984).

Sexual behaviour of ewes.

The word "oestrus" is a Latin version of the Greek "oistros" meaning "gadfly, excitement, or inspiration" and was first used to describe the period of sexual desire in the female by Heape (1900). The German word "brunst" had been used previously for this purpose by Bonnet (1884) who reported that the "brunst" in sheep recurs every 17 days.

Ewes usually reach puberty between 6-9 months when spring born (Foster 1988). Of the farm animals, ewes show least behavioural evidence of oestrus (Fraser 1972). The oestrous ewe will however stand to be mated, rub herself against the ram (Banks 1964), and fan her tail (Banks 1964; Fraser 1968; Grubb & Jewell 1973; Hulet, Alexander & Hafez 1975). Banks (1964) recognised 3 phases of oestrus. The first is a low intensity period. The ewe stands still as the ram approaches but then moves away, stops, and looks over her shoulder at him. This period may last 3-5 hours. From 5-15 hours after the onset of oestrus there is a period of high intensity soliciting by the ewe. She rubs and nuzzles the rams flank and often noses his scrotum whilst standing alongside. This results in both animals circling, and may stimulate the ram. A ewe will often disrupt the courtship activities of other ewes and rams at this time. From 15-28 hours a low intensity period of

oestrus is observed and this wanes to its termination.

When housed, ewes tend to adopt the same places to rest, but when in oestrus they move and lie close to rams (Banks 1964). Observations of field mating have shown that in many cases the oestrous ewe seeks out the ram (Inkster 1957; Lindsay & Robinson 1961; Lindsay 1966). Ram seeking behaviour has been shown to increase in ovariectomised ewes as the dose of administered oestrogen is increased (Lindsay & Fletcher 1972). Pedometric measurements indicate no increase in general activity at oestrus (Lindsay & Fletcher 1972).

If oestrous ewes are provided with different breeds of ram they prefer to mate with their own breed (Lees & Weatherhead 1970).

When more than one ewe is in oestrus, competitive "harem" groups may form around the ram (Mattner, Braden & Turnbull 1967; Tompkins & Bryant 1972). Unlike cattle, there is little evidence of homosexual mounting in all female groups and oestrus is difficult to detect (Kilgour 1985) in the absence of rams.

#### Sexual behaviour of rams.

The sexual behaviour of rams has been described by several authors (Banks 1964; Pepelko & Clegg 1965; Mattner, Braden & Turnbull 1967; Pretorius 1972; Grubb 1974; Hulet, Alexander & Hafez 1975; Blockey 1979 and Fowler 1984). Outside the breeding season Soay rams live in all male groups (Grubb 1974) but these disperse up to 2 months prior to the oncoming breeding season and

contact with females is resumed. When presented with a flock of ewes, in the breeding season, rams preferentially approach oestrous ewes (Lindsay 1965) and procede to "nose" the perineal area "as if to gain olfactory or gustatory information" (Banks 1964). Nosing either the vulva or urine is commonly followed by the flehmen response (figure 1). When performing the flehmen response the rams head is raised to about a 30 degree angle, its external nares are drawn back in a flared position and the upper lip is curled back to reveal the toothless portion of upper jaw. The flehmen posture may be held momentarily or it may be held for up to 2 minutes (Banks 1964). (For a full account of this behaviour see page 35). The ram may then "nudge" the ewe i.e. stand alongside, kick her flank with his nearest forelimb, and then lower the head and utter low pitched guttural bleats. The tongue is commonly flicked in and out of the mouth. If the ewe stands still, the "mount" occurs and ejaculation follows after one or more mounting attempts. Depending on the receptivity of the ewe, one or more of the earlier components of courtship may be omitted.



Figure 1. Rams exhibiting the flehmen response

## CHEMICAL SIGNALS IN DOMESTIC UNGULATES.

### Terminology.

Substances with hormone-like actions which are released from one animal and can influence others were originally called "ectohormones" (Bethe 1932). The term "pheromone" (from the Greek "pherein" - to transfer, and "hormon" - to excite) was first proposed by Karlson & Luscher (1959). The term describes substances secreted into the environment by an individual organism which, when received by another individual of the same species, elicits a specific reaction such as a definite behavioural response or a developmental process. The definition was based on what was known about insect pheromones.

Wilson & Bossert (1963) subdivided pheromones into:

1. Releaser pheromones. A pheromone to which the response is primarily behavioural and immediate.
2. Primer pheromones. A pheromone to which the response is primarily physiological (e.g. endocrine) and longer term.

Bronson (1968) suggested that the term "releaser" pheromone should be changed to "signaller" pheromone, since the former implied a degree of innateness which may not characterise many mammalian systems.

Beauchamp, Doty, Moulton & Mugford (1976) suggested that the word "pheromone" should not be used in mammalian systems. They stated that the term pheromone



implies:

1. Species specificity,
2. A well defined behavioural or endocrinological function,
3. Minimal dependence on learning,
4. One or a few compounds are involved,
5. Uniqueness in producing the response.

It was thought that the term pheromone had been too precisely defined to be accurately applied to mammalian systems. In mammals, compared with insects, chemical signals are much less likely to cause distinctive behavioural responses required by the definition above. Mammalian responses are governed by complex combinations of sensory cues, the animals physiological state and its past experience.

Müller-Schwarze (1977) states that some chemical signals do not stimulate behavioural or physiological responses but provide information which can be recalled later. He called these chemicals "informer pheromones".

The general term "semiochemical" (Regnier 1971, Albane 1984) has been suggested to describe "single or groups of compounds conveying information or otherwise mediating interactions between organisms in the shared natural environment".

So little is known about the nature and identities of putative mammalian pheromones that semantic discussions concerning their description seem premature.

In this work the word "pheromone" is used to

indicate chemical substances produced into the environment which when received by conspecifics cause a rapid behavioural response or a physiological change in the receiver.

Evidence for Pheromones involved in reproduction.  
Female domestic ungulates.

Hart (1987) states "pheromones are assumed to exist in mammals, particularly with the occurrence of estrus (sic) in females. They function presumably to communicate the occurrence of estrus or impending estrus to conspecific males. To date no pheromone from females of any species of farm animal has been chemically isolated and identified."

(i) Ewes.

Olfaction is important in the detection of oestrous ewes by the ram (Lindsay 1965; Fletcher & Lindsay 1968; Fraser 1968). It appears that the ewes genital tract is the source of pheromone which the ram uses to detect oestrous ewes (Kelley 1937; Bland & Jubilan 1987). The results of Stevens, Perry & Long (1982) suggest that urine is an important source of the olfactory stimulus indicating the sexual state of the ewe.

After olfactory bulbectomy rams approach ewes at random whereas intact rams preferentially approach oestrous ewes (Lindsay 1965). It is not known whether this effect is due to an olfactory deficit, a vomeronasal deficit or some other sensory deprivation. The mating ability of anosmic rams is not impaired

(Lindsay 1965; Smith 1975) and they are able to detect oestrus by the characteristic immobility of sexually receptive ewes. Fletcher and Lindsay (1968) confirmed that anosmic rams are capable of detecting and copulating with oestrous ewes. Signoret (1975a) concluded that the ewes immobility is the critical sensory cue to the ram that she is sexually receptive.

Signoret (1976) states that even though a chemical signal from the oestrous ewe is not necessary to ensure copulation, such a signal probably does exist since the levels of mating activity are depressed in anosmic rams and also the normal patterns of courtship behaviour (including flehmen and anogenital sniffing) are absent. Fletcher & Lindsay (1968) studied anosmic, olfactory bulbectomised, rams when joined with groups of ten oestrous ewes. They found that anosmic rams mated with 55% of oestrous ewes under field conditions whereas intact rams mated with 97% of oestrous ewes. This is almost certainly due to poor oestrus detection by anosmic rams since Lindsay (1965) found that when the same time was allowed with a mixture of oestrous and non-oestrous ewes, intact rams approached 80 oestrous ewes and 29 non-oestrous ewes whereas anosmic rams approached 96 oestrous ewes and 98 non-oestrous ewes (i.e. more approaches but less efficient oestrous detection). Smith (1975) described oestrous detection efficiency in terms of the number of approaches divided



by the number of mounts, and found that intact rams achieved 3 approaches per mount whereas rams with olfactory bulbectomies needed 5 approaches per mount. Similar results have been obtained by using topical intranasal anaesthesia to induce anosmia (Rouger 1973). Intact Ile de France rams achieved 6 matings per 10 minute mating test compared with 2 matings for anosmic rams. Intact Prealpes rams achieved 8 matings in the 10 minutes whereas anosmic Prealpes rams achieved only 1 mating, in the same time.

Signoret (1976) concludes that chemical signals from the oestrous ewe do exist, but that their importance is limited. They are not essential for sexual arousal of the ram or for the identification of sexually receptive ewes.

Behaviour studies indicate that ewe urine contains chemical signals. Non-oestrous ewes urinate more frequently than do oestrous ewes when approached by a ram (Geist 1971; Bland & Jubilan 1987). Stevens et al. (1982) have suggested the existence of a "non-oestrous indicating urinary pheromone" to explain the relatively high urination rates of non-oestrous ewes. Ewes show a marked fall in water intake at oestrus (Mitchell 1979) which for Cheviot ewes lasts throughout oestrus and amounts to approximately 27% of normal intake. However the total output of urine falls only slightly at oestrus. It seems likely that the low frequency of urination by oestrous ewes is not simply a manifestation

of decreased urinary output.

(ii) Other female domestic ungulates.

Males of other ungulate species regularly investigate the urine and anogenital areas of females (Grau 1976). This includes horses (Waring, Wierzbowski & Hafez 1975), goats (Shank 1972; Ladewig, Price & Hart 1980) and cattle (Hafez & Bouissou 1975) - review Izard (1983).

The role of olfaction in the detection of oestrous cows by bulls has been controversial. Hart, Mead & Regan (1946) reported that vaginal mucus and urine from oestrous cows attracted and stimulated bulls. Hale (1966) noted that bulls used for semen collection would mount teasers of either sex and concluded that attempts to enhance the sex drive of bulls by olfactory stimuli were unsuccessful. As Signoret (1976) has pointed out, immobilisation seems to be a strong enough stimulus for the release of mounting behaviour by the bull, regardless of sexual odour signal (Rouger 1974). More recently Paleologou (1977) showed that cervicovaginal mucus from oestrous cows contains volatiles which are attractive to bulls. Urine may contain similiar stimuli (Sambraus & Waring 1975). More recently, Blasquez, French, Long & Perry (1988) have demonstrated a correlation between increased bull olfactory behaviours and increased perineal skin gland discharge induced by intradermal injection of adrenaline into the perineal

skin. This finding supports the hypothesis that the perineal skin glands (Blasquez, Batten, Long & Perry 1987; Blasquez, Long, Perry & Watson 1987) are a source of oestrous pheromone in the cow.

Other species have been trained to detect odours associated with oestrus. Both dogs (Akhlebininskii & Ishnutov - cited by Ladewig & Hart (1981); Kiddy, Mitchell, Bolt & Hawk 1978; Kiddy, Conley & Hawk 1980; Kiddy & Mitchell 1981) and rats (Ladewig & Hart 1981) have been trained to demonstrate their discrimination between the odours of urine samples taken from oestrous and non-oestrous cows.

The production of oestrous odours by mares appears to be an aid and not a requirement for oestrous detection. Such odours are similarly unnecessary for arousal of the stallion (Signoret 1976).

Conversely the sow does not appear to produce such odours. Boars are only slightly more attracted to oestrous sows than to non-oestrous sows (Signoret 1970) and actively pursue both.

There is recent evidence that pheromones from female ungulates may have effects on conspecific females. Izard & Vandenberg (1982b) have described a synchronising effect on the oestrous cycles of cows after oro-nasal applications of cervical mucus from other oestrous cows.

## Male domestic ungulates.

### (i) Rams.

Rams appear to produce odours which act as signals to attract ewes. "Ram seeking " by oestrous ewes has been reported by Inkster (1957); Lindsay & Robinson (1961) and Hulet et al. (1962). Fletcher & Lindsay (1968) showed that ewes with olfactory bulbectomies were less able to compete for tethered rams and consequently less ewes were mated compared to intact ewes. Such "ram seeking" behaviour is not due to any general increase in activity at oestrus, as shown by pedometric measurements (Lindsay & Fletcher (1972)).

The effect of the presence of rams on the timing of puberty in ewe lambs is controversial. Dýrmundsson & Lees (1972) reported that the sudden introduction of a ram has a synchronising effect but does not influence the age at first oestrus. Izard (1983) stated " there is limited anecdotal evidence that the ram may have an effect on the attainment of puberty in ewe lambs, but there is no experimental evidence of this so-called ram effect (Dýrmundsson (1981))". However, Oldham & Gray (1984) have demonstrated that the presence of rams can advance puberty, at least in Merino ewe lambs.

When a ram is introduced to a group of post-pubertal ewes, prior to the onset of the breeding season, the first oestrus occurs earlier than it would have done in the absence of rams and subsequent oestrous

cycles are synchronised (Underwood, Shier & Davenport; 1944; Watson & Radford 1960; Martin & Scaramuzzi 1983; Pearce & Oldham 1984). Watson & Radford (1960) showed that neither sight nor contact was necessary for this effect, suggesting a role for chemical communication. Morgan, Arnold & Lindsay (1972) reported that anosmic ewes did not respond to the stimulus as well as intact ewes. Knight & Lynch (1980) demonstrated that rams fleeces were as effective as rams for inducing ovulation and synchronised oestrus, which suggested that the fleece was the source of pheromone. Ram urine had some effect but was a relatively minor source of pheromone. More recently Cohen-Tannoudji, Locatelli & Signoret (1986) have demonstrated that the "ram effect" can occur in sexually experienced ewes with olfactory ablations and concluded that other sensory systems can substitute for olfaction in mediating the induction of oestrus by rams (i.e. sight or LH surges due to learned anticipation).

The continuous presence of rams shortens the duration of oestrus and neither the ewes sight nor physical contact between the sexes is necessary for this effect (Fletcher & Lindsay 1968). However, anosmic ewes respond similarly to intact ewes therefore olfaction is not essential. The response is mediated by unidentified sensory receptors (Aron 1979). The ram accelerates ovulation by advancing the LH surge relative to the onset of oestrus (Lindsay et al. 1975; Signoret 1975b).



Mauléon & Dauzier (1965) have reported a shortening of lactational anoestrous in ewes by the presence of rams (Signoret 1979).

(ii) Other male domestic ungulates.

The only pheromones to be isolated from domestic ungulates are found in boar saliva (Patterson 1968; Booth 1975). 5 $\alpha$ androstene and 3 $\alpha$ androstene are odorous steroids found in high concentrations in adult boar saliva, but only in trace quantities in sow saliva. Either, or both, of these steroids elicits the "standing reaction" from oestrous sows. Perry, Paterson, Macfie & Stinson (1980) showed that the secretions of boar's submaxillary salivary glands were essential for normal oestrous behaviour in gilts. In the absence of the boar not all sows will demonstrate oestrus to the human observer by "standing" when forward pressure is applied to their backs. A commercial preparation of the aforementioned steroids has been produced (Boar-mate, Antec.A.H. International Ltd). When sprayed on the noses of oestrous sows it will potentiate the "standing reaction" (Melrose, Reed, & Patterson 1971) and is useful as an aid to the detection of oestrus, especially when using artificial insemination. High affinity binding receptors for both steroids have been isolated from the olfactory mucosa of the sow (Gennings, Gower & Bannister 1977).

In several species the male is able to hasten and

synchronise puberty (Signoret 1979, Izard 1983).

Kirkwood & Hughes (1979) showed that the introduction of a boar could accelerate puberty in gilts from 139 days of age onwards but not if the boar was introduced before this age. Brooks & Cole (1970) suggested that the delayed and variable response when the boar was introduced before this time was due to conditioning or habituation to his presence. It was subsequently shown that "Boar-mate" could not accelerate puberty but that the odour of pens having contained boars could do so (Kirkwood & Hughes 1980). Kirkwood, Forbes & Hughes (1981) demonstrated that gilts subjected to olfactory bulbectomy at 6-7 weeks of age reached puberty at the same age as intact gilts isolated from boars, but 3 weeks later than intact gilts with experience of boars. Boars less than 6 months old could not produce this effect but boars over 11 months could do so (Kirkwood & Hughes (1981). This is not due to varying testosterone levels as they are the same in both ages of boar.

The urine of a bull has been shown to accelerate puberty in heifers (Izard & Vandenberg 1982a).

In post-pubertal females the presence of males can end seasonal anoestrus. The billy-goat is thought to end seasonal anoestrus and synchronise oestrous cycles in female goats in a similiar manner to that described for rams (Shelton 1980) however the experimental evidence is less clear.

In the sow, 5 $\alpha$ androstene-3-one (Boar-mate) can shorten the length of lactational anoestrus (Hillyer 1976). Sows were weaned at 35 days postpartum and some were sprayed on the nose with "Boar-mate" after 2 days whilst others were similarly sprayed after 2 days and 4 days. The first group took an average of 10 days to come into oestrus while the second group took an average of 9 days. A control group which had no exposure to boars or "Boar-mate" took an average of 27 days to show oestrus (Hillyer 1976).

There is some evidence in cattle that the first oestrus after calving can be advanced by the presence of bulls (Izard 1983-review).

Evidence for the existence of pheromones in domestic ungulates comes essentially from observations of behaviour. The nature of many putative pheromones remains unelucidated.



### CHEMOSENSORY SYSTEMS.

There are five potential systems which may mediate responses to chemical stimuli:

#### THE MAIN OLFACTORY SYSTEM.

The structure and function of the main olfactory system has been reviewed by Allison (1953); Moulton & Beidler (1967); Hare (1975); Dodd & Squirrell (1980) and Lancet (1986). The cellular and molecular aspects of olfactory function have been recently reviewed by Margolis & Getchell (1988).

Within the nasal cavity, the respiratory and olfactory regions can be differentiated macroscopically. The respiratory area is reddish-pink in colour whereas the olfactory epithelium is pigmented and varies in colour according to species. The olfactory epithelium is yellowish in the horse, ox and sheep; olive brown in the goat; brown in the pig and grey in the dog and the cat. It is restricted to the dorsal and caudal part of the nasal cavity and differs from the respiratory areas in being thicker, having Bowmans glands, bipolar sensory cells, and cilia which do not beat rhythmically (unlike respiratory cilia).

The fine structure of vertebrate olfactory epithelia has been described by Moulton & Beidler (1967); Graziadei (1971) and Menco (1980). Kratzing (1970) has described the sheep's olfactory epithelium.

### Sensory cells.

Sensory cells can be identified by their apical specialisations. Each cell has an "olfactory knob" which protrudes above the epithelial surface and carries 40-50 cilia which are given off at different levels. The cilia may be very long and are thought to be the location of olfactory receptor sites (see Lancet 1986 for review). The cells are narrowest proximally and widest at the nucleus.

### Supporting cells.

The apices of supporting cells have a fringe of microvilli which are about 2 $\mu$ m long and 0.15 $\mu$ m diameter. The microvilli mesh with the olfactory cilia. Tight junctions occur between the walls of supporting and sensory cells.

### Basal Cells.

The basal cells are located on the basement membrane and their cytoplasm extends into long irregular processes which form sheaths around the proximal extensions of sensory cells. Basal cells are thought to be the stem cells for the regeneration of olfactory sensory cells.

### Sub-epithelial structures.

There are 2 distinctive features of the sub-epithelial region. The "Bowmans glands" are simple tubular glands which secrete olfactory mucus onto the epithelial surface. The second feature is the abundance

of olfactory nerve fibres. There may be up to 30 unmyelinated axons in a single process of a Schwann cell. Each sensory cell has a proximal extension which is an olfactory nerve axon. This passes through the cribriform plate to the olfactory bulb.

#### The Main Olfactory bulb.

Cajal (1911) classified the olfactory bulb into 7 layers, namely the peripheral neural layer; glomerular layer; mitral cell layer; inner plexiform layer; granular layer and periventricular layer. The central projections of the olfactory bulb have been extensively reviewed (Scalia 1968; Macleod 1971; Leonard & Tuite 1981).

#### Sensory cell regeneration.

Unlike almost all other mammalian neurons the olfactory and VN sensory cells have the capacity to replace themselves periodically and to regenerate after injury (Takagi 1971; Graziadei & Monti-Graziadei 1978; Brunjes & Frazier 1986; Costanzo & Graziadei 1987). However, there is controversy about whether these new connections are functional (Wright & Harding 1982; Butler, Graziadei, Monti-Graziadei & Slotnik 1984). This information is relevant when interpreting reversible lesioning experiments where return to function might be due to new sensory cell populations (see Chapter 6).

### THE VOMERONASAL ORGAN.

The discovery of the vomeronasal organ (VNO) is usually attributed to Jacobson (Cuvier 1811; Jacobson 1813 [1948]) but the organ was noted earlier by Ruysch (1703), a military surgeon, who recorded its presence in a soldier with a facial wound (Wysocki 1979). The VNO is found in every order of mammal with the possible exception of the cetacea (Wysocki 1979). The phylogeny of the organ has been discussed by Parsons (1970) and Bertmar (1981).

The anatomy and physiology of the VNO has been recently reviewed by Johns (1980) and Halpern (1987), but most extensively by Wysocki (1979). More specific descriptions, especially of the anatomy of the VNO in the various domestic ungulates are provided by the short bibliography on page 24.

#### The Vomeronasal Organ of sheep.

The VNO of the sheep (figure 2) was first described in detail by Balogh (1861) and later by Von Brunn (1892), who provided the first illustrations of silver-stained bipolar neurons in the vomeronasal (VN) epithelia of sheep. Simonetta (1932) subsequently produced elegant diagrams of silver stained bipolar neurons (Bielschowsky method) in the VN epithelia of lambs.

Aspects of gross anatomy have been described more recently by May (1964) and Frewein (1972). Bastiaanse (1984) investigated the development of the VNO in lambs

in utero. Kratzing (1971) describes the gross and microscopic anatomy of the sheep VNO in some detail.

Anatomy of the vomeronasal organ in various domestic ungulates - a short bibliography.

<u>Genus.</u>	<u>Authors.</u>
<u>Bos taurus</u>	Jacobson (1813), Broom (1897), Raugé (1893) Goodall (1912), Minett (1926), Frewein (1972), Adams (1986). Taniguchi & Mikami (1985)
<u>Bos taurus and Bos indicus</u>	Jacobs et al (1981)
<u>Bubalis bubalis</u>	Kumar et al (1981),
<u>Ovis aries</u>	Jacobson (1813), Balogh (1861), Von Brunn (1892), Raugé (1893) Goodall (1912), McCotter (1912), Broman (1920), Simonetta (1932), May (1964), Kratzing (1971) Frewein (1972), Bastiaanse (1984),
<u>Sus scrofa</u>	Jacobson (1813), Broom (1897), Goodall (1912), Minett (1926), Matthay (1968),
<u>Equus caballus</u>	Jacobson (1813),  Broom (1897), Goodall (1912), Kerkhoff (1924), Minett (1926), Frewein (1972), Lindsay & Burton (1983) Taniguchi & Mikami (1985)
<u>Camellus dromedarius</u>	Minett (1926), Nagpal et al (1988)



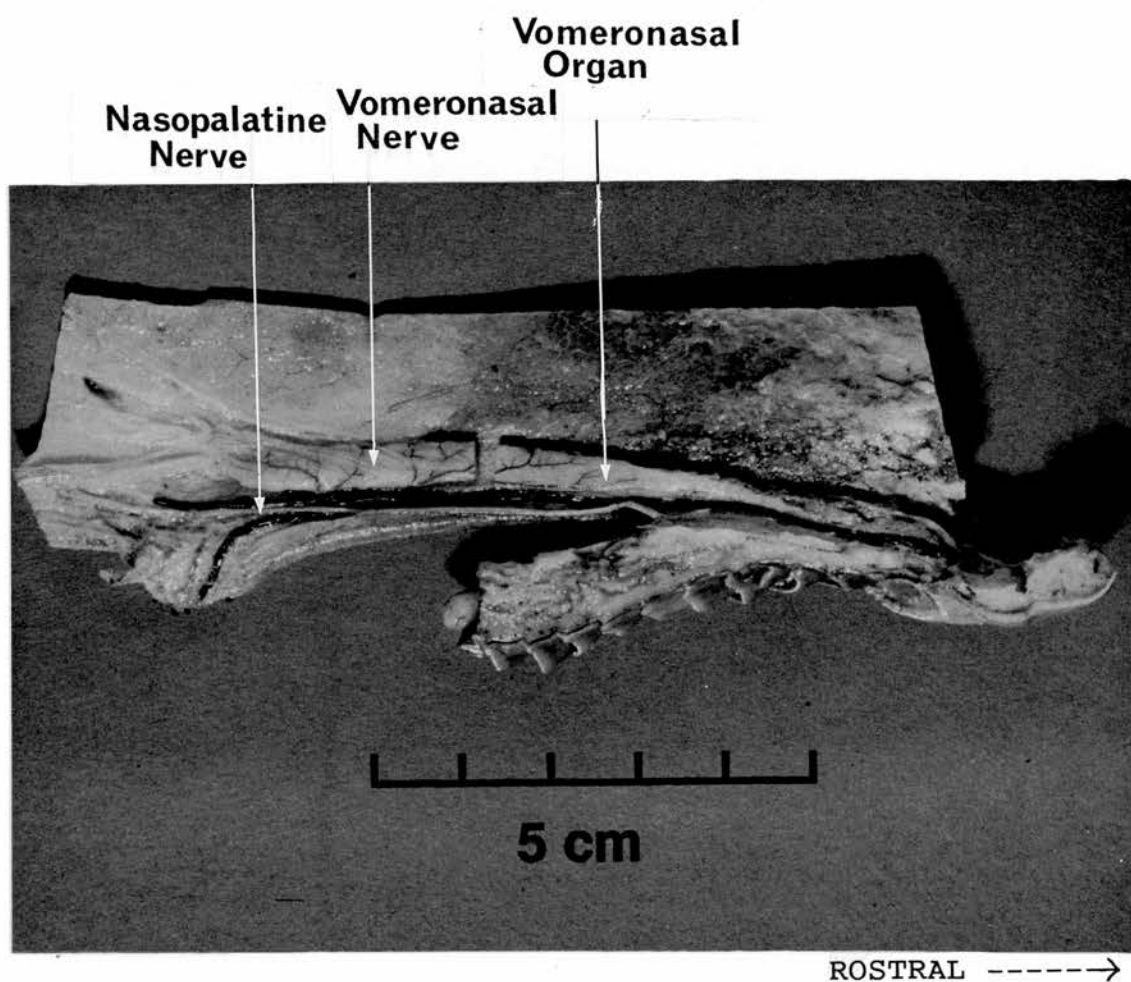


Figure 2. Dissected specimen showing medial aspect of the left vomeronasal organ of a ram. Latex arteriogram shows related arterial blood supply.

a). Embryology. (Bastiaanse 1984).

The VNO is first recognisable in the sheep at around day 25 of gestation. From about day 28, two ectodermal invaginations arise on the septum of the cavum nasi and start to become tubular and blind ending. The epithelium then changes to pseudostratified. By day 34 the whole organ is tubular and has a slit-shaped lumen. The lumen is C-shaped by day 70 and by parturition two distinct types of cells line the lumen. These types are the "sensory" cells and the "respiratory" cells.

(b) Gross anatomy. (Kratzing 1971).

In the adult sheep the VNO consists of 2 epithelial tubes, 6-8 cm long, lying at either side of the base of the nasal septum in the rostral half of the nasal cavity. The tubes are blind ending and the posterior end reaches to the level of the 4th premolar tooth (Frewein 1972). Each tube opens anteriorly via a VN duct into the ipsilateral nasopalatine canal (Goodall 1912) which allows communication with either the mouth or the nose. However, Kratzing (1971) precludes any contact with the nasal cavity due to the "sharp angle at which the VNO enters the duct". Each tube is surrounded by a VN cartilage which is incomplete dorsally.

(c) Microscopic anatomy.

The lumen is C-shaped (Kratzing 1971), being displaced medially. The convexity of the lateral aspect of the lumen is due to thickened connective tissue,

glands and blood vessels (Broman 1920 - cited in Hamlin 1929). The epithelium over this area is pseudostratified consisting of ciliated cells, basal cells and cells without apical specialisation which may be supporting or secretory.

Typical sensory epithelium is restricted to the ventral aspect of the concave portion of the lumen. Three cell types are present - sensory cells, supporting cells, and basal cells. The most numerous are supporting cells which reach from the basal lamina to the epithelial surface. The apical surface is usually smooth but may have a few microvilli. The sensory cells are large and their nuclei form an epithelial zone above the basal cells but beneath the supporting cell nuclei. The apical surface carries numerous long (13-15 $\mu$ m) microvilli and a microprocess (Kratzing 1971) which is 2-3 times larger in diameter than the microvilli. There appears to be only one microprocess per cell. The cell's axon passes through the basal lamina as an axon of the VN nerve and joins the accessory olfactory bulb (McCotter 1912). The central projections of the vomeronasal system in the sheep have not been described. In laboratory rodents (Johns 1980) the accessory olfactory bulb projects to the corticomedial part of the amygdala (Scalia & Winans 1976) which does not receive direct input from the main olfactory bulb (Heimer 1975). The amygdala is traditionally associated with social and



emotional responses and has projections to preoptic and hypothalamic areas implicated in social and sexual behaviours. The VNO, unlike the main olfactory system, does not have widespread connections with thalamic and neocortical areas associated with cognitive processing. The central projections of both systems appear therefore quite distinct.

#### Functions of the VNO.

##### (a) Historical.

Various functions have been ascribed to the VNO. Jacobson (1813 [1948]) thought the organ to be secretory, whilst Kolliker (1877) described it as a self-smelling system. It was also described as a rudimentary accessory olfactory organ (Röse 1893), a specialised olfactory organ (Cajal 1904), a device for smelling liquid borne odours (Broman 1920; Kerkhoff 1924), an organ to detect smells during feeding (Young 1950; Negus 1958; Romer 1962), and an organ utilised in the chemoreception of sex-related odours (Estes 1972; for review see Wysocki 1979).

##### (b) VNO mediated neuroendocrine responses in laboratory rodents.

There is direct evidence, from experiments with laboratory mammals, indicating that the VNO is important in mediating chemical signals between individuals, especially those concerned with reproduction. These experiments are based on removal or lesioning of the vomeronasal sensory system (Wysocki & Meredith 1988).

Male mice or their odours significantly affect the reproductive physiology of female mice (and vice versa). Lesions to, or removal of, the vomeronasal organ in mice has the following effects:

i) Prevents the acceleration of puberty and induced synchronisation of oestrus in grouped females by male odours (Lomas & Keverne 1982).

ii) Prevents the blocking of pregnancy by the odours of strange males (Bellringer<sup>et al.</sup> 1980; Lloyd Thomas & Keverne 1982).

iii) Prevents the increased aggression of maternal females to intruder males (Bean & Wysocki 1986- cited in Wysocki & Meredith 1988).

iv) Drastically reduces the reflex release of Luteinising hormone (LH) and the subsequent testosterone surge (Wysocki 1983), in males exposed to female urine (Coquelin, Clancy, Macrides, Noble & Gorski 1984). Aggression and sexual behaviour are also reduced to some extent (Clancy, Macrides, Gorski & Noble 1984).

v) Virtual elimination of ovulation induced by the bedding of males (Johns, Feder, Komisaruk & Mayer 1978, Johns 1980).

In female rats VN lesions have the following effects:

i) Reduction of the time taken for virgin rats to show maternal behaviour (Fleming, Vaccarino, Tambosso & Chee 1979).

- ii) Prevention of the surge of LH in females exposed to males or their odours (Beltramino & Taliesnik 1983).
- iii) Elimination of advancement of puberty by male odours (Sanchez-Criado 1982).

Lesions of the VNO have been shown to increase some aspects of maternal behaviour e.g. pup carrying in hamsters (Marques 1979).

(c) VNO mediated sexual behaviour of laboratory rodents.

Removal of the VNO in sexually experienced male mice caused significant mating deficits whereas in sexually inexperienced mice VNO removal virtually eliminated mating (Clancy, Macrides, Singer & Agosta 1984; Bean & Wysocki 1985). Sexually experienced male hamsters have no mating deficits following VNO removal but most inexperienced males have some deficits (Meredith 1986). VNO removal in guinea pigs was even less effective (Beauchamp, Martin, Wysocki & Wellington 1982). The importance of the VNO in male mating behaviour seems to diminish higher up the evolutionary scale. Olfactory ablation (removing the input from both main and accessory olfactory tracts) does not prevent rams or billy-goats from mating (Lindsay 1965; Ladewig & Hart 1980). The VNO is vestigial in humans (Johnson, Josephson & Hawke 1985).

It is concluded by Wysocki & Meredith (1988) that the VNO mediates some pheromonal responses in naive animals but with experience these responses may be triggered by conditioned cues detected by other sensory

systems such as the main olfactory epithelium. The VNO may be stimulated by volatile or non-volatile chemicals. The presence of mucus in the VNO seems to favour stimuli which are mucus soluble.

(d) Role of the VNO in domestic ungulates.

Very little experimental evidence of a role for the VNO exists in domestic ungulates. Klemm, Sherry, Sis, Schake & Waxman (1984) attempted to block stimulus access to the VNO of cattle at the oral opening of the nasopalatine canals. The feed intake and weight gain of treated cattle did not change but the social hierarchy of treated groups of cattle did change. The authors conclude that the VNO has a role in the aggressive behaviour contributing to the social hierarchy in cattle.

Recent work by Cohen-Tannoudji, Lavenet, Locatelli, Tillet & Signoret (1989) has shown that, unlike the rodent, the LH surge in anoestrous ewes exposed to rams, or their odours, is not mediated by the VNO. VNO deprivation was achieved either by sectioning of the VN nerves or by cautery of the entire organ. The main olfactory system appears to mediate the "ram effect".

(e) Filling and emptying mechanisms of the VNO.

Chemical stimuli must be actively drawn into the VNO to react with chemoreceptors. Mikalkovics (1899) suggested that smooth muscle tissue (between the lumen and the lateral cartilage) dilated the lumen and allowed

the organ to fill. The "wide veins" in the wall compress the lumen so as to empty the organ (Hamlin 1929). Broman (1920) described large muscular venous sinuses in the lateral wall and suggested that they might act as a pump to fill and empty the VNO. Hamlin (1929) reported that intravenous adrenaline acts to expel the contents of the VNO in the rabbit. Estes (1972) reviewed potential filling mechanisms. He suggested that during flehmen the male ungulate closes the external nares and inhales - causing a stream of air to pass upwards through the nasopalatine canals. The VNO empties, it is suggested, by the operation of the Venturi effect sucking out the contents. The resulting vacuum is supposed to result in refilling at the end of inspiration.

It has been established experimentally that a sympathetically controlled vasomotor filling mechanism exists in the anaesthetised hamster (Meredith & O'Connell 1979), cat (Eccles 1982) and ram (Bland & Cottrell 1989).

Other behavioural actions such as tongue movements in the bovine (Jacobs, Sis, Chenoweth, Klemm, & Sherry 1981) and "head bobbing" in guinea pigs (Beauchamp, Wellington, Wysocki, Brand, Kubie & Smith 1980) have been implicated as acts which facilitate the entry of chemical stimuli into the VNO.



#### OTHER POTENTIAL CHEMOSENSORY SYSTEMS.

There are 3 other recognised potential chemosensory systems about which very little is known.

##### Nervus Terminalis.

The Nervus terminalis has been reported in the ovine embryo (Larsell 1918), the adult sheep (Simonetta 1932) and the cow (Larsell 1918). The nervus terminalis (Locy 1905) connects the midline forebrain with the olfactory and respiratory mucosa (Read 1908; Larsell 1918, 1950). The nerve was described as chemoreceptive, based on the presence of free nerve endings in the nasal epithelium. Branches of the terminal nerve traverse the nasal septum with the VN nerves (Bojsen-Møller 1975). Demski & Northcutt (1983) electrically stimulated the nerve in male goldfish and observed a stimulation dependant release of sperm. Wirsig & Leonard (1985) have demonstrated mating deficits in male hamsters with bilateral lesions of the nervus terminalis. As has been pointed out by Wysocki & Meredith (1988) these deficits are very similiar to those produced by VNO or olfactory lesions and it has become important to discover the exact role of this nerve in odour perception.

##### Septal organ.

There are no reports of the presence of a septal organ in the sheep. The role of the septal organ (Masera 1943) in chemoreception has not been properly elucidated. The organ is a localised island of sensory

cells similiar to olfactory sensory cells and has been identified in laboratory rodents (Graziadei 1971). It is located either side of the nasal septum at a narrow passage between the nasal cavity and the nasopharyngeal canal (Leonard & Tuite 1981). Axons from the organ connect with the caudal olfactory bulb (Bojsen-Møller 1975).

Trigeminal nerve endings.

Despite little anatomical study, trigeminal nerve fibres are known to respond to odours in the tortoise and rabbit (Tucker 1963; Stone & Rebert 1970; Tucker 1971). The contribution of these inputs is presently unclear.

### FLEHMEN.

The term "flehmen" was first used by Schneider (1930). The derivation of the word is an enigma (Estes 1972), however its use has persisted, unlike Schneider's (1930, 1931) synonymous, and much clearer term "rumpfegebarde" (to turn up ones nose). Many synonymous terms have subsequently been used: "lipcurl" (Geist 1971); the "artiodactyl grimace" (Cowan 1956); "la moue" (Schloeth 1958); the "olfactory reflex" (Fraser 1968) and "testing" (Estes 1972; Lindsay & Burton 1983).

#### Ontogeny.

##### (i) Sheep.

Flehmen in lambs was reported by Geist (1971) and has been observed in a 9 week old male Barbary sheep in response to its mothers freshly voided urine (M.J.Blissitt - personal observation).

Orgeur & Signoret (1984) investigated the initiation and occurrence of sexual play in lambs and found that lambs of both sex will "nose", "mount", "nudge" and flehmen from a few days old. The frequency of sexual play peaks at about 4-5 weeks of age and then returns to a low level until puberty when it disappears in females, but in males becomes more selectively orientated towards females. These findings support the earlier results of Thwaites (1982) who showed that male-like patterns of sexual behaviour, including flehmen, are present in young lambs of both sexes and this contrasts with the situation in the adult where

such behaviours are absent in the female. Lambs of both sexes flehmen to other lambs, of either sex, with equal frequency (Thwaites 1982). Donchin, De Vane & Caton (1987) reported flehmen in lambs, from 2 days - 9 weeks of age, following intracisternal administration of a methionine enkephalin analogue (Metkephamid - Sigma Ltd). Pre-treatment with naloxone blocked flehmen in 6 out of 7 lambs and the authors conclude that opioid peptides may be involved in the expression of flehmen in lambs.

(ii) Ontogeny in other ungulates.

Crowell-Davis & Houpt (1985) studied Welsh ponies (Equus caballus) and observed flehmen in colt foals from 2 days of age and in filly foals from the first day of life. Flehmen frequency in colts peaks at 1 - 4 weeks of age and then declines to 20 weeks. Tyler (1972) observed flehmen in 1 day old foals.

Flehmen has been observed in 7 day old Bos indicus calves (Reinhardt 1983). The frequency of flehmen increases after 16 weeks which coincides with the first "sexual interest". Scheurman (1975) records flehmen in calves of both sexes of Mithan (Bibos frontalis - Lambert 1837). Müller-Schwarze (1971) observed flehmen in 12 week old Black-tailed deer and Shank (1972) observed flehmen in goat kids. Dagg & Taub (1970) recorded flehmen in a Giraffe calf (Giraffa camelopardis) but concluded that this was an abnormal

behaviour , probably as a result of captivity.

It is concluded that young ungulates are able to flehmen, but that flehmen prior to puberty is a manifestation of play behaviour and has no apparent sexual motivation.

#### Flehmen in the ram.

There is a body of evidence, from studies of ungulate behaviour, suggesting that flehmen assists in the detection of putative pheromones which indicate the ewes sexual state.

Careful studies have described both the occurrence of ram flehmen following "nosing" of oestrous ewe genitalia and the relationship of flehmen in response to urination by ewes at different stages of sexual receptivity. These studies have included wild sheep such as the Bighorn (Geist 1971); feral Soay sheep (Grubb & Jewell 1973); as well as British domestic sheep (Stevens et al. 1982; Tompkins & Bryant 1974; Bland & Jubilan 1987).

#### Occurrence of flehmen in rams.

Geist (1971) observed that flehmen was characteristic of sexually mature rams, although it was performed rarely by yearling rams. Geist (1971) states that there is no competition between rams for ewe urine. Several rams may stand side by side and simultaneously lipcurl where a ewe has urinated. Rams may also flehmen in response to their own urine or the urine of other rams.



Geist (1971) recorded the frequency of ram flehmen to oestrous and anoestrous ewes relative to the ewes urination frequency. Anoestrous Stones ewes and yearling females, in late autumn, urinated to 71% (671 observed courtships) and 73% (227 observed courtships) of courtships respectively, resulting in an average of 30-40 flehmens per 100 courtships. In contrast, oestrous Stones females showed no urination responses and provoked only 7 lipcurls from courting rams (144 observed courtships). Geist (1971) concludes that the urination response of the ewe may be an adaptation to get away from the ram, since the ram usually nuzzles the urine and lipcurls for long enough to allow the ewe to leave the scene.

Tompkins & Bryant (1974) compared aspects of behaviour in 2 groups of ewes. One group was in normal oestrus and the other group was treated with progesterone sponges to synchronise oestrus. The behaviour of rams to the 2 groups of ewes was also observed. During the 24 hours before the onset of "standing oestrus" the frequency of flehmen by rams was greater in response to the treated ewes. Ram flehmen was positively correlated with ewe squatting ( $P < 0.01$ ) and negatively correlated with aspects of the ewes behaviour such as tail fanning, looking over the shoulder, and active soliciting (all components of oestrous behaviour). These results show that ram flehmen occurs

at stages of the ewes oestrous cycle which do not coincide with oestrus.

This is in agreement with Signoret (1975a) who showed that the frequency of ram flehmen was greater ( $P < 0.05$ ) to progesterone treated ewes than to ewes induced into oestrus with either testosterone or oestrogen. It seems that ram flehmen occurs more frequently when the ewe has higher endogenous progesterone levels i.e. the non-oestrous state.

Stevens et al. (1982) found that the urination rate of oestrous domestic ewes was very low in spite of intensive ram courtship and that flehmen was not a reliable indicator of oestrus. Again it was found that rams flehmened to non-oestrous ewes and that flehmen was elicited by urination. Oestrous ewes urinated less frequently to the approaching ram than non-oestrous ewes ( $P < 0.001$ ). Flehmen was however observed being performed by rams in contact with oestrous ewes.

Jubilan (1983) and Bland & Jubilan (1987) have further clarified the occurrence of ram flehmen. The frequency of ewe urination and flehmen are strongly correlated. Flehmen to ewe urine occurs least often in the presence of the oestrous ewe, with higher frequencies and little variation on other days of the cycle. This is due to the low urination frequency of oestrous ewes. Flehmen by rams to the ewes vulva is of short duration and most frequent the day before oestrus. The latter findings are interpreted as being due to the

onset of standing oestrus, at which time the ram is allowed by the ewe to get near the "preferred site" which is the vulva. The short duration of flehmen to the vulva is explained in terms of the stimulus-receptor theory of olfaction which states that the saturation of sensory receptors is faster the more concentrated the stimulus. The duration of flehmen should be inversely related to stimulus concentration. It is suggested that the vulva is a more concentrated source of chemical stimulant than urine. The authors conclude that these findings support the hypothesis that the role of flehmen in the ram is to confirm the reproductive state of the ewe, with the object of identifying the time of oestrus or sexual receptivity. The decreased incidence of flehmen at oestrus is interpreted as not detracting from this role because the oestrous ewe can communicate her receptivity to the ram by more immediate and powerful cues than those reputed to involve flehmen, the most significant of which is likely to be her willingness to stand for service at this time.

#### Critique.

Our understanding of flehmen in the ram has been hampered by errors of interpretation and the application of observations from other species which have subsequently been shown not to apply. One such assumption was that flehmen in rams would occur most frequently when investigating oestrous ewe urine.

Müller-Schwarze (1979) states that Tompkins & Bryant (1974) demonstrate "rams show more flehmen in response to urine of oestrous ewes than of those not in heat." This is not the case. Clarke (1977) interprets Hafez, Cairns, Hulet & Scott (1969) as stating that " the oestrous ewe often squats and urinates at the initiation of her courtship display to attract the attention of the ram" but what Hafez et al. (1969) actually state is that rams flehmen to oestrous urine and that if the sexes have been separated , the oestrous ewe frequently urinates when the ram is introduced. Whitten (1985) states that "a ewe will urinate for a ram whenever he stamps his foot or nuzzles her rump, and he receives urine directly into his nares."

These statements need careful reinterpretation in the light of recent work. The females of many species do urinate with increasing frequency as they come into oestrus i.e. the bitch, the sow, the mare, the Black rhino, various bovids, felids and primates (Fraser 1968; Ewer 1968; Tembrock 1968; Michaels & Keverne 1968 - cited in Estes 1972). The ewe is a notable absentee from this list.

#### Other male ungulates.

##### (i) Goat.

Like sheep, flehmen in male goats is seen much more frequently in males than females and seems to be under some degree of androgenic control. Castration reduces the incidence of flehmen in tropical male goats (Hart &

Jones 1975). Ladewig et al. (1980) reported that flehmen in male goats occurs more frequently during copulation than preceding it. It has also been shown that olfactory ablation "virtually eliminated" flehmen in male goats (Ladewig et al. 1980). This agrees with the findings of Lindsay (1965) in rams. Also like sheep, flehmen is seen in all male groups in response to the urine of other males or their own urine (Shank 1972; O'Brien 1982).

(ii) Stallion.

Flehmen in response to olfactory stimuli has been described in the horse and donkey (Lindsay & Burton 1983). Tyler (1972) describes flehmen by stallions to the genitalia of oestrous mares; by both sexes, of all ages, to urine; and also "imitatively". In the latter case a horse performs flehmen when it sees another horse flehmen. The reason for this is not clear. Houpt, Panzer, Ryan, & Rini (1985) reported that stallions flehmen to oestrous and dioestrous mare urine odour with similar frequencies and that this frequency is greater than that for ovariectomised, anoestrous or male urine odours. Marinier, Alexander & Waring (1988) also found no differences in the frequency, latency to respond, or duration of flehmen to oestrous and non-oestrous urine or vaginal secretions of mares (samples presented in isolation from the donor mare). However, Houpt & Guida (1984) point out that when the oestrous mare is present there is an increased incidence of flehmen by stallions



to urine because oestrous mares urinate more frequently than dioestrous mares. Staulbaum, Ekong, Houpt & Meinwald (1983) have fractionated mare urine and conclude that a stable, organic, component of oestrous mare urine with low volatility actively elicits the flehmen response.

(iii) Bull.

Reinhardt (1983) states that cows cause bulls to flehmen more often when they are in oestrus than when they are not and that sexually mature bulls flehmen most frequently. Hradecký, Sis, & Klemm (1983) observed flehmen by a bull, in response to 3 cycling cows, every day for 9 months. Flehmen was not restricted to oestrous periods. Forty six percent of observed flehmen responses occurred within oestrus and fifty four percent outwith oestrus. It was concluded that flehmen was "very loosely" related to events during the cows oestrous cycle. However more flehmen responses occurred around oestrus (day -3 to day +1) than on other days ( $P < 0.01$ , t-test).

(iv) Boar.

Despite the common belief that boars do not flehmen, this behaviour has been reported by Martys (1977) and by Sambraus (1981).

Ewes and other domestic female ungulates.

Flehmen is not exclusively a male behaviour, although there is no doubt it occurs more frequently in the male. Bland & Jubilan (1987) report flehmen in a ewe

in response to ram urine. Flehmen in the ewe has hardly been considered, but it is known that female domestic ungulates, including the ewe, may flehmen either to their newborn young or birth fluids. Mares commonly flehmen to their birth fluids or the anogenital region of their newborn foals (Fraser 1968; Tyler 1972; Rossdale 1975; Crowell-Davis & Houpt 1985). Altieri & Müller-Schwarze (1980) report flehmen in female Black-tailed deer. Female goats are reported to flehmen to the urine and genital areas of both sexes and to the birth fluids of their newborn (Ladewig et al. 1980; Hart 1983). Shank (1972) never observed flehmen in feral female goats but O'Brien (1982) comprehensively describes flehmen in female feral goats (Capra hircus). He observed that females flehmened in response to their own and unrelated neonates, and to amniotic fluids and membranes. Flehmen to neonates was restricted to females with offspring under 4 days of age. In 14 flehmen incidents where the mother flehmened to unrelated neonates, she moved away from the youngster on 6 occasions and on 8 occasions became aggressive towards it. Maternal flehmen is reported as being observed commonly in Musk-oxen (Lent 1974) , mares (Fraser 1968), and ewes (Banks - cited in Lent 1974).

Donchin et al. (1987) have recently described flehmen in two ewes during prolonged uterine contractions. Kendrick, Keverne & Baldwin (1987) have

reported flehmen in ewes during 10 minute periods of vaginocervical stimulation with a plastic probe (to imitate the passage of a lamb through the birth canal). Interestingly there were increased levels of oxytocin in cerebrospinal fluid (CSF) following vaginocervical stimulation. Increased levels of CSF oxytocin stimulates maternal behaviour including olfactory investigation of the lamb (Kendrick et al. 1987).

The relationship between the VNO, Flehmen  
and Pheromone detection.

Estes (1972) concludes that "on the basis of anatomical, histological and ethological studies" a connection between flehmen and the vomeronasal organ may be suspected. Fraser (1968) surmised that as substances eliminated in the urine can induce flehmen and because urine can contain breakdown products of female hormones " it is a reasonable assumption that oestrous cycle phasing may be recognisable to the male animal by odour testing urine. The occurrence of flehmen prompted speculation that flehmen may be a means of facilitating the entry of pheromones into the male vomeronasal organ (Estes 1972). Dagg & Taub (1970) disagreed with the proposed functional connection between flehmen and the vomeronasal organ. They disputed Knappe's (1964) proposed filling mechanism on the basis of their dissections of ruminant heads. These studies convinced them that flehmen could not alter the tissues around the vomeronasal organ. Dagg & Taub (1970) concluded that flehmen trapped odours in the nose so that they could be more thoroughly evaluated by the main olfactory epithelium. Schneider's (1930) observation, that horses stopped breathing when they performed flehmen, was used as supporting evidence. Lindsay & Burton (1983) state that breathing does occur during flehmen in horses and Ladewig & Hart (1980) state that during flehmen in goats

breathing is unchanged whether following a period of rest or exercise.

Estes (1972) suggested that "urine testing" was of fundamental importance in determining the receptive state of females, but agreed that the involvement of flehmen in vomeronasal organ function remained to be established. He suggested that flehmen functioned mainly to close the external nares so that aspiration of air through the nasopalatine ducts might bring odorants to the vomeronasal organ. Ladewig & Hart (1980) stated that goats do not close their external nares during flehmen.

There is a paucity of experimental evidence from domestic ungulates to support the hypothesis that flehmen facilitates the entry of pheromones into the VNO for detection. The work of Ladewig & Hart (1980) and Melese d'Hospital & Hart (1985) in goats, provides the best evidence to date.

Ladewig & Hart (1980) injected a fluorescent dye (sodium fluorescein) into the mouths of male goats and evoked flehmen by presenting a dish of urine to the animals. After flehmen the animals were killed and the maxilla removed and placed in liquid nitrogen. The VNOs of all animals were examined for the presence of dye. Comparisons were made between animals which had performed flehmen and those which had not. The time from administration of dye to tissue immersion in liquid nitrogen ranged from 2.5 - 6 minutes. Animals which had



flehmened had dye in the posterior part of the VNO (reported to be the site of a receptor epithelium by these workers). Animals which had not flehmened either had no dye in the VNO or dye in the anterior or middle parts of the VNO. Fluids can therefore pass from the mouth through the nasopalatine canals and into the VNO without flehmen. Difficulties of interpretation arise with these results owing to the time span between flehmen and examination of the VNO post-mortem. The mechanism by which dye entered the VNO may be unrelated to flehmen. A negative pressure in the VNO may have arisen, after death, which could draw dye into the organ. Such a negative pressure has been demonstrated in the ram (Bland & Cottrell 1989) and can be activated by stimulation of the sympathetic nerves to the VNO or by increased levels of circulating adrenaline. Ladewig & Hart (1980) conclude that during flehmen some mechanism occurs which pulls material from the basal part of the nasal cavity or the nasopalatine duct into the VNO. The flehmen posture is supposed to coincide with this mechanism but the significance of the actual posture is unknown.

To overcome the problems of sampling VNO contents after flehmen, Melese d'Hospital & Hart (1985) conducted chronic cannulations of the VNO in goats. The same type of dye mixed with either water or urine was administered into the mouths of male goats. The contents of the VNO were sampled after flehmen (or after 1 minute

if no flehmen occurred). Some dye diffused into the VNO of animals which did not flehmen. Significantly more dye was found in the organs of animals which had flehmened than in the organs of those which had not. However, in animals which did not flehmen, similiar amounts of dye were found in the VNOs regardless of whether urine/dye or water/dye was administered into the mouth. Therefore urine per se, does not provoke active VNO uptake without flehmen. The authors conclude that it is likely that flehmen somehow produces an alteration in the nasopalatine canals which facilitates transport of substances to the nasal cavity where they are further transported into the vomeronasal organ "by means of a vascular pump surrounding the organ". Such a pump has been demonstrated for other species (see page 31) including the sheep (Bland & Cottrell 1989).

There is no direct evidence of a functional connection between the VNO and flehmen in rams.

From the foregoing review it is apparent that many questions remained unanswered in relation to the role of odour communication in ovine reproduction. It was unclear whether rams could detect oestrous ewes by the odour of the ewes urine. If this was the case then it would be necessary to determine the location of chemoreceptors which mediated this discrimination in order to determine if the VNO or the main olfactory epithelium mediated the discrimination.

#### AIMS OF THIS WORK.

1. To determine if rams can perceive a difference in the odours of oestrous and non-oestrous ewe urine.
2. To determine the respective roles of the VNO and the main olfactory epithelium in mediating the detection of urine volatiles associated with female sexual receptivity.
3. To observe the incidence of flehmen in rams during olfactory discriminations between oestrous and non-oestrous ewe urine.
4. To determine the effect of selective chemosensory deprivation on the incidence of flehmen.

In order to achieve these aims domesticated rams were selected as experimental animals and the technique of operant conditioning was employed to demonstrate their ability to perform simultaneous two-choice olfactory discrimination experiments.

## CHAPTER 2 MATERIALS AND METHODS.

### INTRODUCTION.

Skinner (1938) recognised two classes of response to a stimulus by an animal, those that are elicited and those that are emitted. Responses elicited by known stimuli were classified as "respondents". The constriction of the pupil to bright light is an example of a respondent. The second class of "emitted" responses were designated as "operants". These responses are voluntary behaviours. Operants usually acquire a relationship with a prior stimulus but are not elicited by them as respondents are in true reflexes (Hill 1965; Reynolds 1968; Bower & Hilgard 1981).

In operant conditioning the frequency with which a particular operant occurs can be increased if the operant is followed by the presentation of a reinforcing stimulus, such as a reward. This differs from the classical conditioning of reflexes (Pavlov 1927) where no voluntary actions and no rewards are involved per se.

New operant behaviour can be created by a process known as "shaping". By reinforcement of simple animal behaviour which progressively approaches the desired final behaviour, it is possible to produce more complex responses to the same stimuli. This principle is the essence of most animal training methods.

In the work presented here rams were trained to press switches with their noses to demonstrate their discrimination between certain odours. The operant in



this work was therefore "switch pressing" and the rams were conditioned to perform the operant in the presence of odour stimuli by the presentation of a food reward immediately following the operant.

This technique has been used successfully to demonstrate sensory discriminations in various species of domestic livestock. Olfactory discriminations have been demonstrated in pigs (Meese, Conner, & Baldwin 1975), sheep (Baldwin & Meese 1977), goats and calves (Baldwin 1977). Visual discriminations have been investigated in goats (Baldwin 1979), sheep and calves (Baldwin & Start 1981). Sheep have been shown to adapt well to training procedures (Bremner 1980). Operant responding has also been reported in dairy cows and horses (Myers & Mesker 1960; Whittlestone, Mullord, Cate & Waite 1975).

#### ANIMALS.

##### Rams.

Four adult stud rams (2 Suffolk and 2 Cheviot) were trained to use the olfactory discrimination apparatus. Experiments were conducted between 9.00 a.m. and 11.30 a.m. during the natural breeding season of sheep in Great Britain (October to March). Rams were housed at night in individual pens and put out to grass after the daily experiment. Hay and supplementary concentrate ration were fed indoors and water was available ad libitum. The rams were identified as A,B,C, and D.



### Ewes.

Five ewes (3 Cheviot and 2 Welsh x Suffolk, numbered 180, 181, 182, 197 and 198) were used as urine donors. All ewes were showing normal oestrous cycles. The ewes were housed at a separate site from that occupied by the rams and were checked daily for oestrous behaviour with one of two vasectomised teaser rams housed in separate pens nearby.

### APPARATUS.

#### Experimental Crate.

The apparatus was designed to demonstrate olfactory discrimination abilities in rams and was based on the original description by Baldwin & Meese (1977). Experiments were conducted in a purpose built crate (figure 3). The crate was 2 metres long, 90cm wide and had solid sides 1.2 metres high. A tray underneath a mesh floor collected urine and faeces. Rams entered the crate by a ramp which lifted up to form the rear door and was secured by steel pins.

A wooden panel at the front of the crate offered rams the choice of two 10 cm square sprung switches beneath two odour nozzles (figure 4). Below the two switches was a centrally positioned food bowl into which the food reward of 10g whole oats could be delivered. A hopper attached to the rear of the wooden panel supplied the bowl through an opening in the panel.

#### Odour delivery system.

The aim of these experiments was to assess the

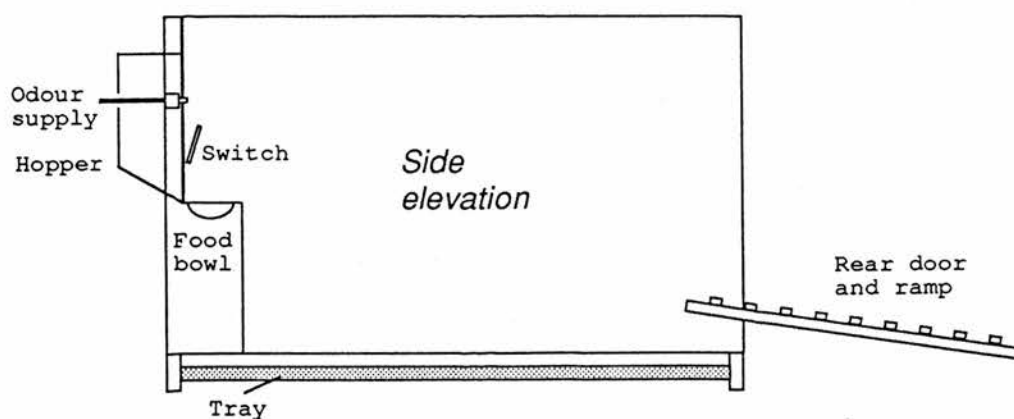
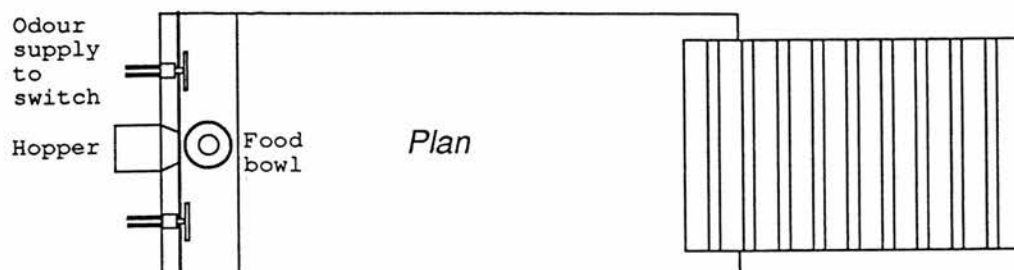


Figure 3. Sheep Crate.

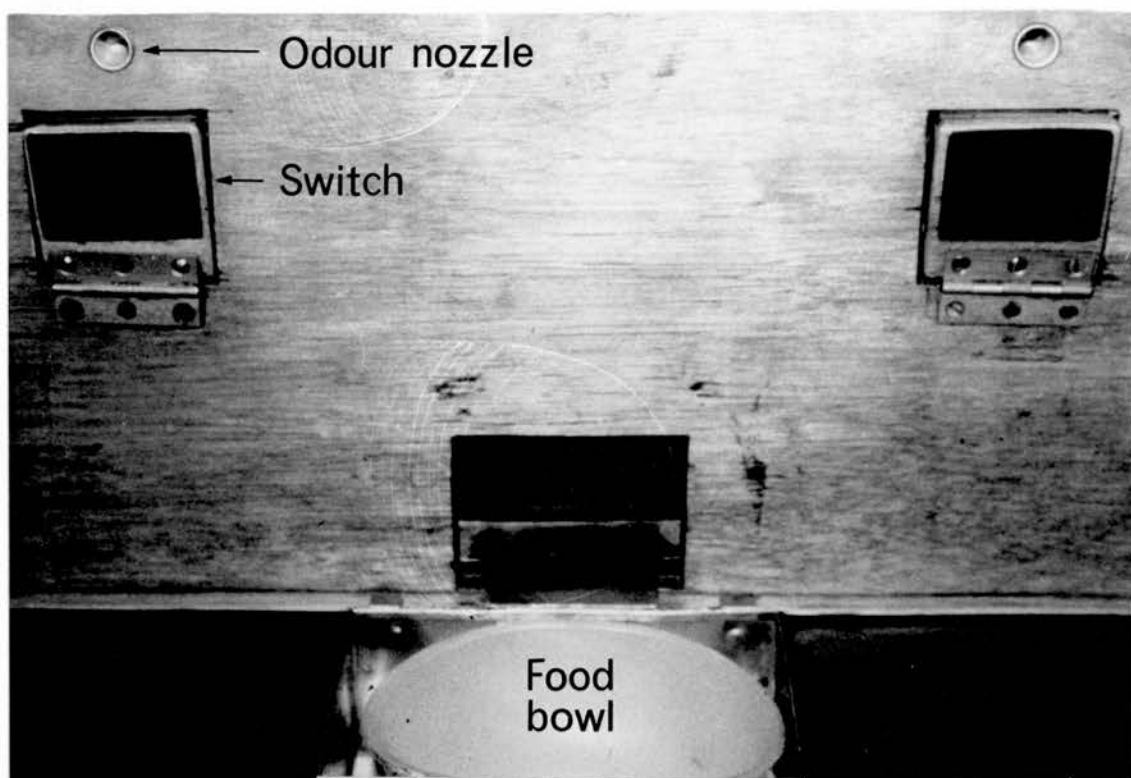


Figure 4. Position of odour nozzles, switches and food bowl at the front of the sheep crate.

ability of rams to discriminate between the odours of various ewe urine samples. Two glass flasks, each containing a 5ml urine sample were placed in a waterbath at 39°C, the ewes body temperature. An airstream was passed over the urine samples and delivered to the odour nozzles above the switches at the front of the crate. Teflon tubing was used to direct the odours to the odour nozzles. The odorous airstreams entered the crate at a nozzles at a flow rate of 1 litre min<sup>-1</sup> and a velocity of 0.59 msec<sup>-1</sup>. Each of the two urine samples was usually taken from one oestrous and one non-oestrous ewe. When oestrous urine odour was delivered to the left odour nozzle, non-oestrous urine odour was delivered to the right odour nozzle and vice versa. This was accomplished using solenoid valves at the odour nozzles. Figure 5 shows the two flow circuits used to direct the odours to the odour nozzles. The air which carried the odours was pumped into the system from outside the laboratory and therefore varied in temperature and humidity according to atmospheric conditions at the time of the experiment. The parts of the apparatus which carried odours were cleaned between experiments with an "odourless" detergent (Decon 90, Decon Laboratories Ltd). Figure 6 shows the mechanism of the odour nozzle valves and their control by two solenoid valves.

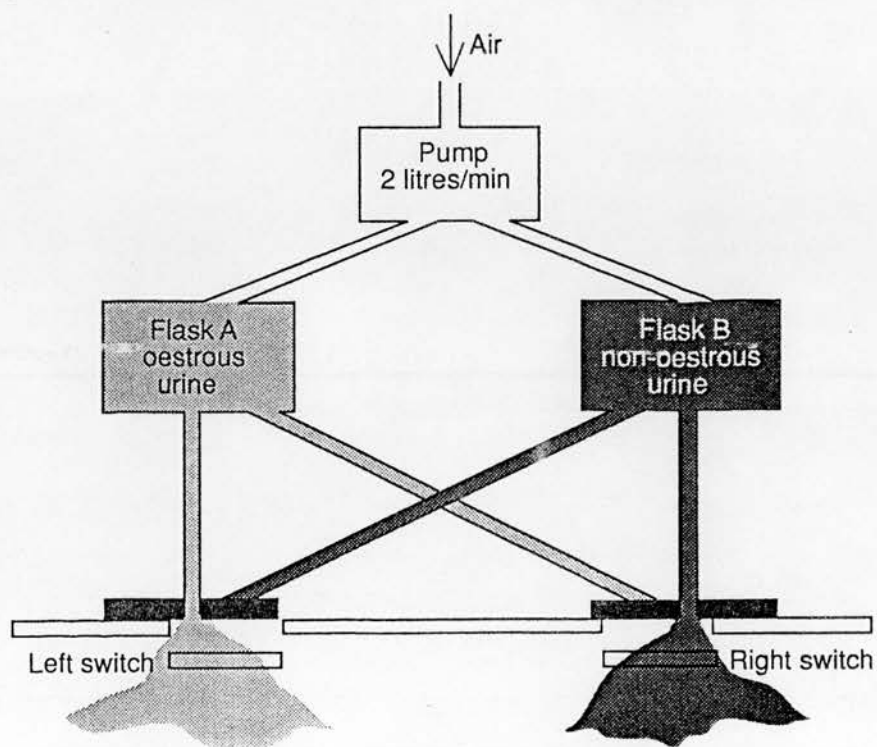
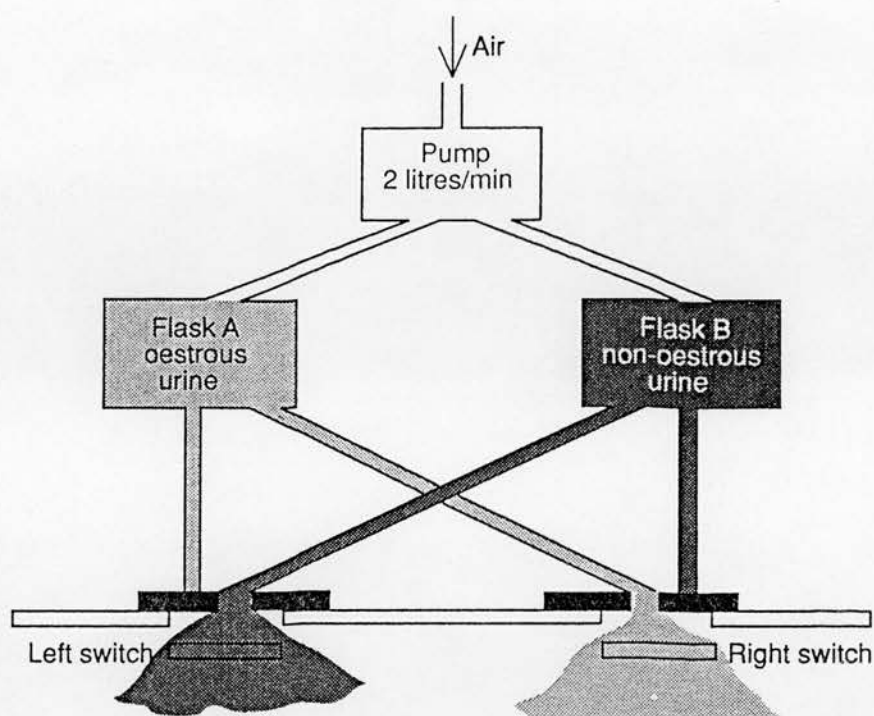


Figure 5. Two circuits used to deliver the odours to the odour nozzles.



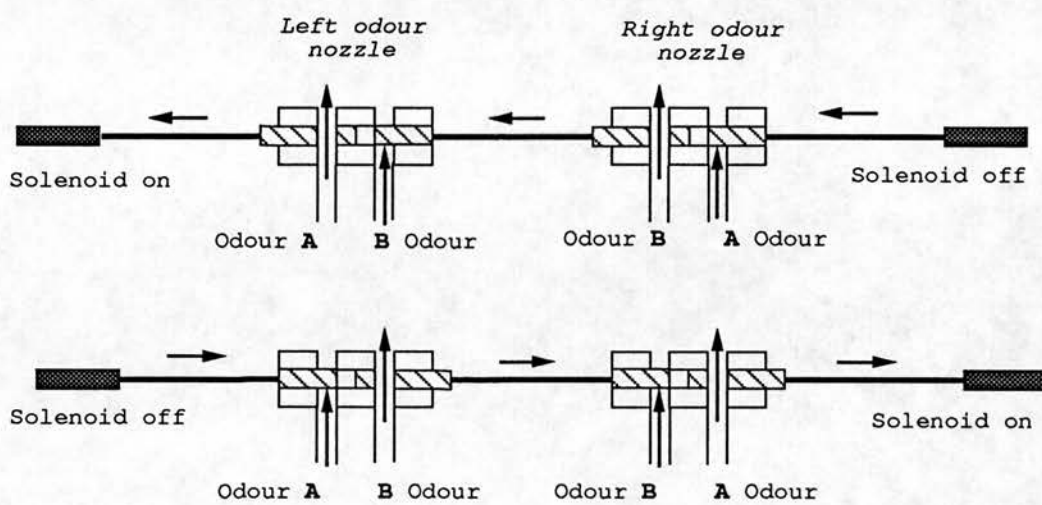


Figure 6.  
Diagram showing the odour nozzle valves

#### TRAINING AND PROCEDURE.

The rams were trained to discriminate between non-oestrous ewe urine odour and "no odour" (an airstream passed over water). Oestrous urine odour was substituted for the water when experiments commenced. Rams were trained to this standard in 8 weeks. The rams were initially trained to indicate their discrimination between odours by pressing the switch beneath the odour nozzle producing<sup>defrosted</sup> non-oestrous urine odour (the positive discriminative stimulus or S+) to obtain a food reward of 10g whole oats. Water "odour" was the negative discriminative stimulus or S-, and presses on the switch beneath this odour nozzle were unrewarded and counted as errors. Non-oestrous urine was initially used as S+ because it was easier to collect than oestrous urine. The collection of non-oestrous urine did not require long and unpredictable periods waiting for ewes to urinate. (The use of a metabolism crate, or cannulation of the urinary tract in order to collect urine was precluded because of the likely contamination of urine samples obtained in this way). An experiment consisted of 50 consecutive choices between the two odours. The odours were alternated from side to side according to the rules of Gellerman (1933) and Fellows (1967). These rules describe orders of alternating stimuli which should be used in simultaneous two choice discrimination experiments to minimise the effects of inherent

position preferences.

Rams were trained to work on a so-called "fixed ratio" schedule of 4 (FR4). Having determined which of the two odours offered was S+, rams pressed the associated switch 4 times to receive the food reward. The fixed ratio schedule of reinforcement was used because at small ratios it is rapidly attained and produces a stable and high rate of responding (Honig 1966). Reynolds (1968) states: "On an FR schedule each response brings the number of responses emitted one step closer to the number in the presence of which a response is reinforced. Each response becomes a conditioned reinforcer of the previous response and a discriminative stimulus for the next. The sequence of responses within each ratio is thus a chain which runs off at a constant rate after the first response".

FR4 is preferable to FR1 because it reduces the tendency to attempt random pressing strategies to solve the discrimination and encourages decision making before the animal starts to press. FR4 also ensures that the ram spends more time pressing switches and less time eating.

Rams were trained to this level using the training programme listed below. A manual hand-held switch was used initially to reward rams at will. Rams were rewarded for entering the crate and the following programme was commenced when they were acclimatised to the crate:

Ram Offered one switch only.

1. Reward for putting nose near switch
2. Reward for touching switch with nose.
3. Reward for pressing switch with nose once.
4. Reward for pressing switch with nose twice.
5. Reward for pressing switch with nose four times.
6. Reward for pressing switch with nose four times (S+ flowing).

Automatic crate mechanism introduced.

Offer ram 2 switches.

7. Reward for pressing switch associated with S+ four times.
8. Alternate S+ left and right, reward only presses associated with S+ switch.
9. Introduce S- odour. Reward only presses at FR4 made on switch beneath S+. S- and S+ moved from side to side, between choices according to the Gellerman (1933) or Fellows (1967) series.

The oats were delivered within 0.1 seconds of the ram making the fourth press on the correct switch.

ERROR SCORES.

The rams ability to discriminate between odours was quantified using a system of error scores (Baldwin & Meese 1977). This system supposed that if the rams were not discriminating between the odours they would either press 4 times or 8 times depending whether their first choice was correct. (This assumed that rams were

maximally efficient and would change from the incorrect to the correct switch as soon as the fourth press on the incorrect switch failed to produce the food reward). Rams would therefore press on average 6 times per choice and, since the daily experiment consisted of 50 choices, would press 300 times. Of these presses, 200 would be correct since to complete the experiment this was the minimum number of presses required. Therefore if the ram was not discriminating between the two odours, the expected chance level of errors (incorrect presses) was 100. Below this number rams could be said to be discriminating in terms of pressing the switch associated with S+ rather than the switch associated with S-.

In practice it was not always the case that rams moved directly from the incorrect switch to the correct switch as soon as the fourth press on the incorrect switch had proved unproductive. The calculated chance error level was therefore set at a strict level.

#### AUTOMATION OF APPARATUS.

##### Counter box.

The number of presses made on each crate switch was recorded by microswitches and displayed at a counter box. The counterbox displayed three sets of figures (figure 7):

1. The cumulative total presses on each crate switch was constantly displayed (Display A).
2. The fixed ratio in use (FR4) was set using thumbwheel



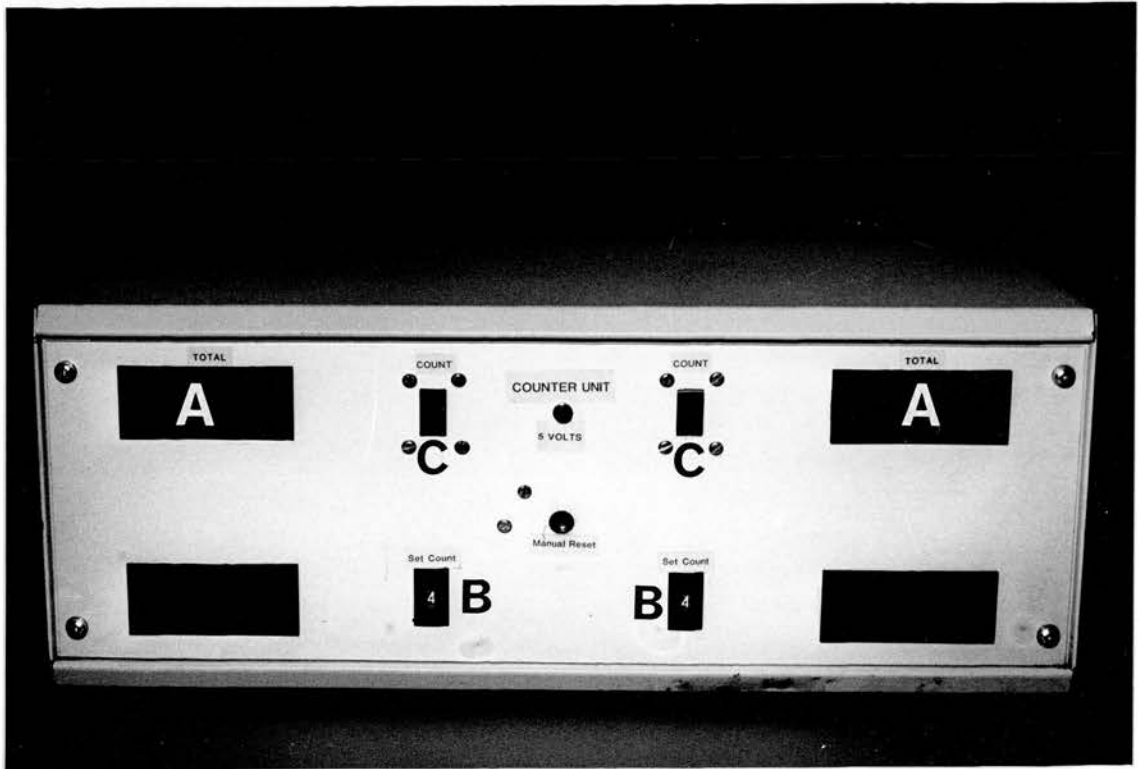


Figure 7. Counterbox.

Display A - Cumulative total presses

Display B - Fixed ratio in use.

Display C - Presses on each switch for each choice.

counters and this was permanently displayed (Display B).

3. The number of presses the ram made on each crate switch for each particular choice was displayed (Display C). This number enabled the experimenter to observe how the ram was performing for any one discrimination test. The numbers shown on Display C were automatically reset to zero before each new choice was offered. When the number display on the crate switch associated with S+ (Display C) equalled the number of presses on the preset F.R. (Display B) then the ram had made a successful choice. A relay then activated one of the hopper solenoids and 10g oats was delivered to the ram. Display C for the switch associated with S- would only show incorrect presses for each choice. By observing this display over the 50 choices, the distribution of errors over the 50 choices could be recorded.

#### The Hopper.

A hopper was constructed which held 1kg whole oats. Two 12V solenoids were positioned at either end of a shuttle delivery box at the bottom of the hopper (figure 8). By supplying 12V to each solenoid alternately, the contents of the hopper were dispensed in 10g quantities. The hopper was activated when the digit display on the preset FR counter (Display B) equalled the number of presses on the crate switch associated with S+ (Display C).

As the hopper shuttle moved backwards and forwards a projecting arm activated a microswitch secured to the

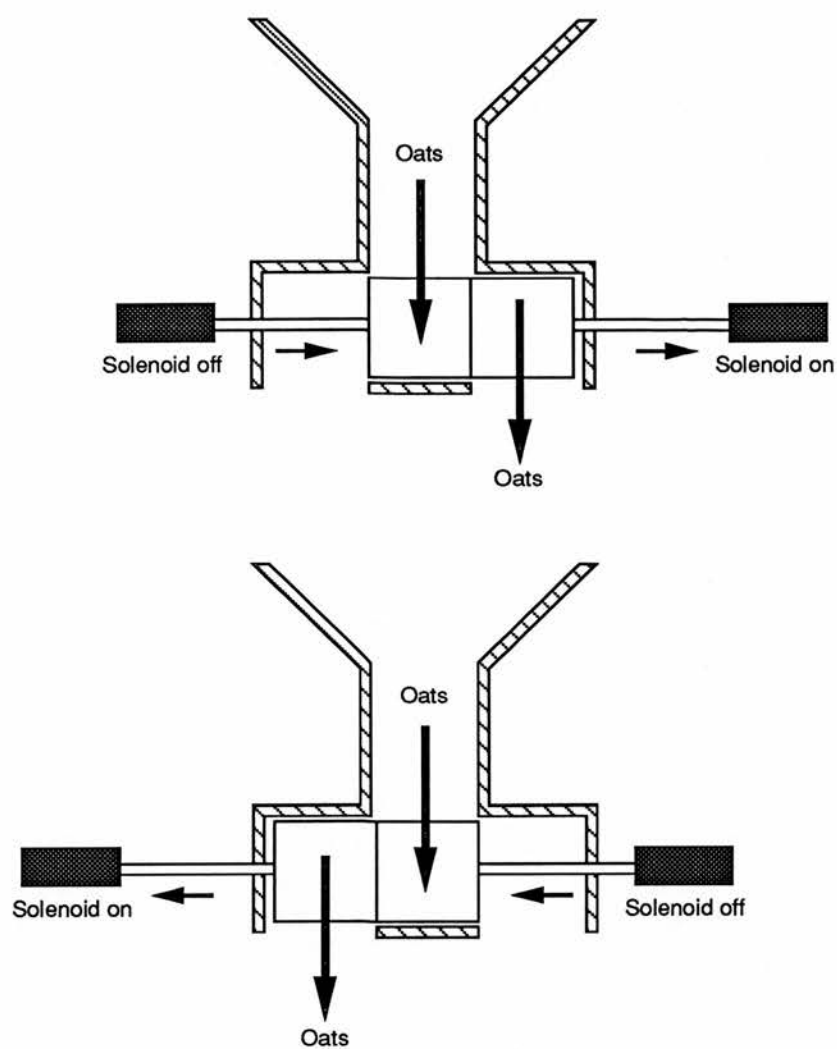


Figure 8. Hopper delivery system. 2 solenoids activated in turn dispense 10g whole oats from hopper.

body of the shuttle casing. The 5V pulse from this microswitch had two functions.

1. It provided a feed-back to the counterbox which reset Display C to zero, ready for the next choice.
2. It provided a further pulse via a Transistor-transistor-Logic (TTL) input at an interface (see next section) which caused the microcomputer to read the next member of the series of 50 choices and to set the crate odour valves accordingly.

#### Computer and Interface.

The mechanisms of odour delivery, hopper activation, and switch counting were controlled by a BBC microcomputer and a Unilab interface (model type 532.001). Purpose written software was produced (Appendix A) which enabled the computer to operate four relays which controlled the electrical power to solenoid valves at the crate. A "TTL" input at the interface provided a feedback circuit which provided the signal to change the relays after each successful choice.

The computer programme offered the operator a menu of choices. When the experimental procedure was selected, the series to be used was read and if the first member of that series was "left" then "PROCLEFT" in the computer programme was initiated. Similarly if "right" was the first member then "PROCRIGHT" in the programme was commenced. (Left and right in these cases refer to the crate switch which was to receive S+ for that particular choice.) The command to move on, read

the second member of the series, and set the apparatus accordingly, was provided by a 5V pulse from the hopper-activated microswitch via a "TTL" input at the interface.

#### Output Control.

Each of the four relays at the interface contained a single pole changeover contact capable of switching 1 amp at 24 Volts. In this work 12V solenoids were used to control the apparatus. The relays were controlled by the codes written at lines 1670 and 1790 of the computer programme (Appendix A). The code used was ?&FCC0 and the numbers following the code when converted to binary are as follows:

	4	3	2	1	RELAYS
240	1	1	1	1	0 0 0 0
247	1	1	1	1	0 1 1 1
249	1	1	1	1	1 0 0 1
254	1	1	1	1	1 1 1 0

Each of the last four binary digits controlled one relay as shown. Digit 1 turned relays "ON" and digit 0 turned relays "OFF". Therefore the four codes had the following actions:

240	All relays OFF.
247	1,2,3, ON. 4 OFF.
249	1 and 4 ON. 2 and 3 OFF.
254	2,3,4, ON. 1 OFF.

Relay 1 switched 12 Volts to each hopper solenoid alternately.



Relay 2 supplied 12 Volts to the left or right odour nozzle solenoids to direct S+ either left or right (and vice versa for S-).

Relay 3 When "ON" enabled the right switch to activate the hopper and when "OFF" enabled the left switch to activate the hopper.

Relay 4 supplied 12 Volts to either the left or right "dummy" solenoids. These solenoids had no function other than to produce the same sound as the odour nozzle solenoids. The "dummy" solenoids were activated whenever the odour nozzle solenoids were not i.e. whenever the odours were delivered to the same sides of the crate for successive choices. The purpose of this system was to cancel auditory cues which the rams could use to help complete the odour discrimination experiments.

#### Inputs.

The hopper-activated microswitch initiated both feedback circuits:

1. To reset Display C for both crate switches.
2. To cause the computer programme to read the next member of the series used in each experiment and set the odour nozzles accordingly. Lines 1700 and 1710; and 1820 and 1830 monitor this input continually for "PROCLEFT" and "PROCRIGHT".

The operant conditioning of rams - A video (Appendix B).

The video presentation included as Appendix B

demonstrates the apparatus in use. The four rams are shown performing olfactory discrimination experiments. The arrangement of the apparatus and the ram housing areas are also shown.

#### Data analysis.

The following statistical tests were used:

##### 1. Students t-test.

This test was used to determine the significance of the difference between the means of the error scores achieved by four rams on different days or using different odour combinations.

##### 2. Significance tests using the Binomial distribution.

This test was used to determine the significance of the ram' ability to choose the S+ odour instead of the S- odour in the first choice of successive experiments (see page 79 - learning sets). If rams chose randomly the probability of being correct or incorrect was 50% or 0.5. The probability of choosing correctly for "c" occasions is  $0.5^c$ .

The relative expected frequency "P" of being correct on "c" out of "N" occasions is given by:

$$P = 0.5^c \times \frac{N!}{c!(N-c)!} \quad \text{where} \quad \frac{N!}{c!(N-c)!} \quad \text{is the binomial coefficient.}$$

The value of "P" is given in each experiment as:  
 $P > 0.05$  (not significant) or  $P < 0.05$ ;  $P < 0.01$ ;  $P < 0.001$   
 (increasing levels of significance e.g. less likelihood of results being due to chance).

CHAPTER 3. OLFATORY DISCRIMINATION BETWEEN DEFROSTED  
OESTROUS AND NON-OESTROUS URINE  
ODOURS FROM THE SAME EWE.

INTRODUCTION.

The first aim of these experiments was to determine if rams could detect a difference between the odours of oestrous and non-oestrous urine collected from the same ewe. As it was impossible to collect both fresh oestrous and fresh non-oestrous urine from the same ewe at the same time, a method of urine storage was required. Previous workers have used urine which has been frozen and thawed as the olfactory stimulus. This method was adopted in these experiments.

The second aim was to establish if freezing and thawing ewe urine changed its odour as perceived by rams. Experiments 1, 2 and 4 were conducted in the breeding season of 1987-88 and experiment 3 in the season of 1988-89.

EXPERIMENTS.

Urine samples.

The first day of standing oestrus was nominated as day 1 of the oestrous cycle. Oestrous urine was collected on this day using a polythene bag, with a glass jar insert, attached to the peri-vulval wool with clips. Vasectomised rams were not allowed to mate with oestrous ewes until after the urine sample had been taken. Urine was either used fresh, or frozen to  $-30^{\circ}\text{C}$  in inert 5ml containers (Vacutainer). Fresh urine was

used within one hour of collection and stored for this interval in similar containers maintained at 39° C, the ewe's body temperature.

"Non-oestrous urine" was defined as urine from day 9 of the oestrous cycle. Day 9 was chosen as the standard for non-oestrous urine samples because it was mid-cycle and representative of the luteal phase. The ewes were tame and the urination response which would normally be elicited by the approaching ram could be evoked by human approach. It was possible therefore to collect day 9 urine by "free catch" into a glass or polythene beaker. The urine was then either used fresh or frozen for storage as described above.

Experiment 1: Discrimination between the odours of oestrous and non-oestrous urine which was fresh or frozen/thawed.

Seventeen daily olfactory discriminations were conducted over a total period of twenty two days. On each day S+ and S- <sup>had been</sup> collected from the same ewe. The experiment consisted of three trials as follows:

Trial 1. S+ frozen/thawed non-oestrous urine odour.

S- fresh oestrous urine odour.

Trial 2. S+ frozen/thawed non-oestrous urine odour.

S- frozen/thawed oestrous urine odour.

Trial 3. S+ fresh non-oestrous urine odour.

S- frozen/thawed oestrous urine odour.

Details of the order in which the trials were

undertaken and the collection dates of urine samples used are given in Table 1. In each experiment at least one of the odours had not been tested previously. These odours are marked with an asterisk in Table 1.

Experiment 2: Effect of freezing and thawing non-oestrous urine.

The purpose of this experiment was to determine whether freezing and thawing non-oestrous ewe urine changed its odour, as perceived by rams. This was suggested by the results of experiment 1. A urine sample in excess of 10 ml was taken from a non-oestrous ewe (day 9 of the oestrous cycle). 5ml was used fresh (S-) and 5ml was used after freezing to  $-30^{\circ}\text{C}$  and thawing (S+). Urine from two ewes (180 and 181) was tested on separate days.

Experiment 3: Effect of freezing and thawing oestrous urine.

The rams were retrained to use frozen/thawed oestrous urine odour as S+ and the ability of the rams to discriminate between fresh and frozen/thawed oestrous urine odour was tested. 10ml of oestrous urine was collected from two ewes (180 and 181). 5ml was frozen/thawed and used as S+, and 5ml was used fresh as S-.

Experiment 4: Control.

The same odour was delivered to both sides of the crate simultaneously. Frozen/thawed non-oestrous urine



TABLE 1. URINE ODOURS PRESENTED TO RAMS IN  
OLFACTORY DISCRIMINATION EXPERIMENT 1.  
SOURCE, NATURE AND ORDER OF PRESENTATION.  
(\* - PREVIOUSLY UNTESTED URINE)

DAY	TRIAL	DONOR EWE	DATE OF SAMPLE NON-OESTROUS URINE S+		DATE OF SAMPLE OESTROUS URINE S-	
1	1	181	5/2/88	} frozen/ thawed	13/2/88*	} fresh
2	1	180	5/2/88*		14/2/88*	
3	1	182	5/2/88		15/2/88*	
4	2	181	5/2/88	} frozen/ thawed	13/2/88*	} frozen/ thawed
5	2	180	5/2/88		14/2/88*	
6	2	182	5/2/88		15/2/88*	
9	3	181	21/2/88*	} fresh	13/2/88	} frozen/ thawed
10	3	180	22/2/88*		14/2/88	
11	3	182	23/2/88*		15/2/88	
12	2	181	21/2/88*	} frozen/ thawed	13/2/88	} frozen/ thawed
13	2	182	23/2/88*		15/2/88	
14	1	183	18/2/88*	} frozen/ thawed	26/2/88*	} fresh
18	1	181	21/2/88		2/3/88*	
19	1	182	23/2/88		3/3/88*	
20	2	181	21/2/88	} frozen/ thawed	2/3/88*	} frozen/ thawed
21	2	182	23/2/88		3/3/88*	
22	2	183	18/2/88		26/2/88*	

odour was used. For each of the 50 choices, the switch which would provide the food reward when pressed was varied according to the Gellerman (1933) series. The purpose of this experiment was to ensure that no confounding stimuli were being used by the rams to complete the experiment. If confounding stimuli were being used ( e.g. the noise of the odour valves) then rams would still have been able to achieve error scores below chance levels despite S+ and S- being the same odour.

## RESULTS.

### Experiment 1: Discrimination of oestrous and non-oestrous urine which was fresh or frozen/thawed.

Rams could consistently discriminate between the odours of oestrous and non-oestrous urine, collected from the same ewe, when at least one of the samples had been frozen and thawed for storage. This was true when S+ was frozen/thawed non-oestrous urine odour and S- was either fresh (trial 1) or frozen/thawed (trial 2) oestrous urine odour. In trial 1 a mean error score of  $30 \pm 4.5$  (S.E.) was achieved compared with  $19 \pm 2.5$  in trial 2 (figure 9).

When S+ was changed to fresh non-oestrous urine odour (trial 3), very high initial error scores were obtained. The mean error score for all three days of trial 3 was  $120 \pm 48.4$  (S.E.), but the combined mean error score for day 2 and day 3 was  $36 \pm 6.8$  (S.E.) (figure 9). The daily error score for all four rams

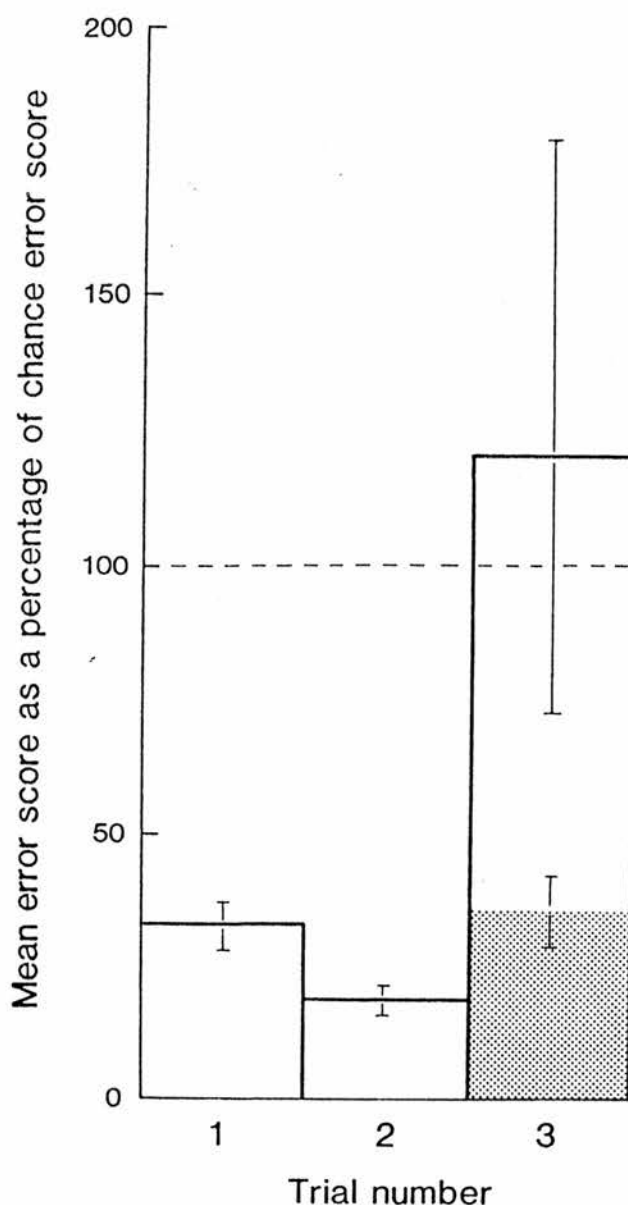


Figure 9. Mean error scores ( $\pm$  S.E.) achieved by rams discriminating between oestrous and non-oestrous ewe urine odours.

Trial 1: S+ Defrosted non-oestrous urine.

S- Fresh oestrous urine.

Trial 2: S+ Defrosted non-oestrous urine.

S- Defrosted oestrous urine.

Trial 3: S+ Fresh non-oestrous urine.

S- Defrosted oestrous urine.

---- = Chance error level.

Hatched area represents mean error score for days 2 and 3 only of trial 3.

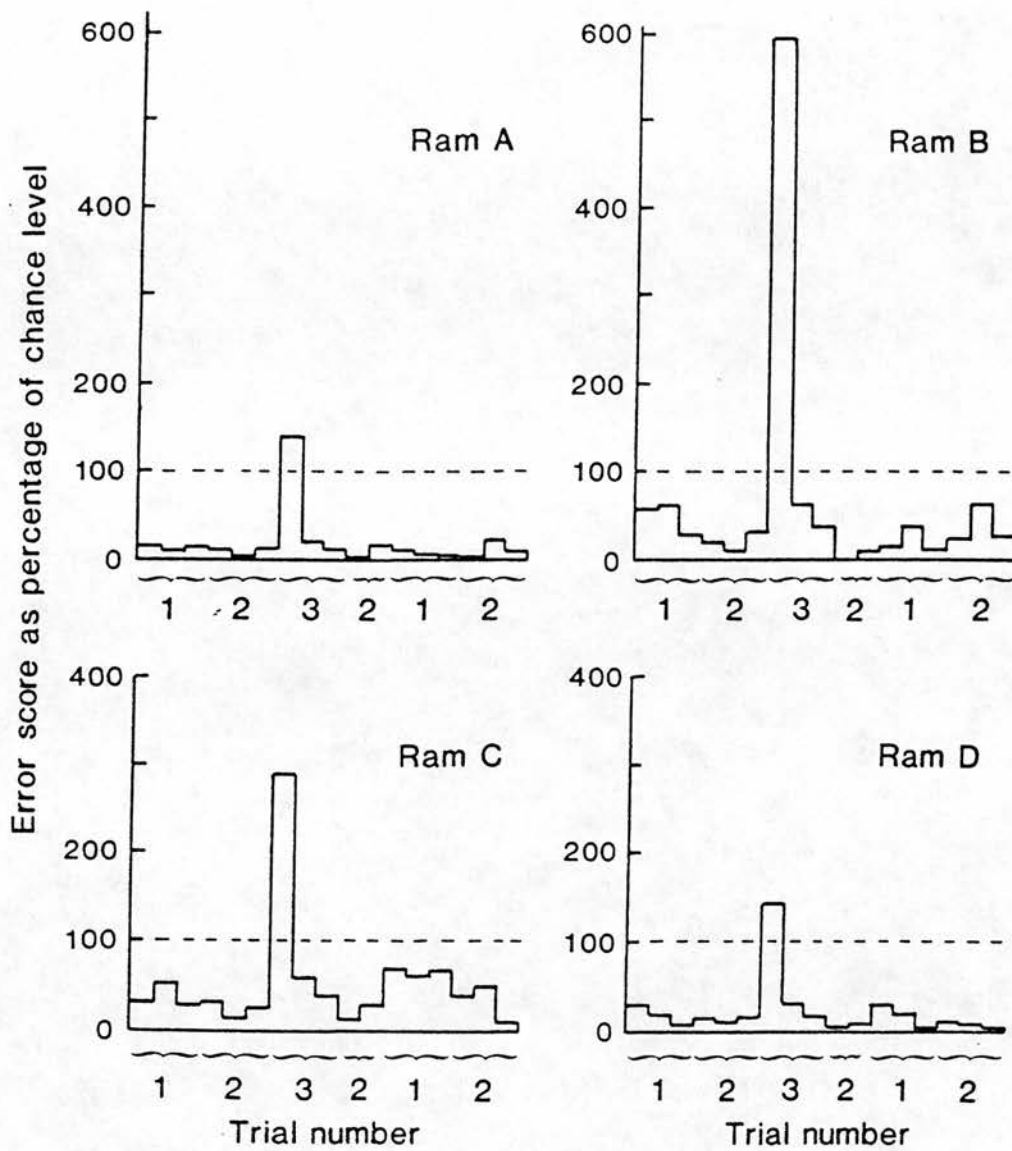


Figure 10. Daily error scores for four rams during 17 olfactory discrimination experiments using oestrous and non-oestrous ewe urine.

Trial 1: S+ Defrosted non-oestrous urine.

S- Fresh oestrous urine.

Trial 2: S+ Defrosted non-oestrous urine.

S- Defrosted oestrous urine.

Trial 3: S+ Fresh non-oestrous urine.

S- Defrosted oestrous urine.

- - - - = Chance error level.

(figure 10) show that the high error score in trial 3 was due to a poor performance by all rams on the first day only i.e. the rams first exposure to fresh non-oestrous urine odour. On the first day Ram B and Ram C pressed more often on the switch associated with S- than on the switch associated with S+ for the duration of the experiment despite not being rewarded. Rams A and D pressed S+ and S- an approximately equal number of times. A marked unconditioned aversion to S+ in trial 3 was thus demonstrated by two rams. The low error scores on days 2 and 3 of trial 3 (figure 10) demonstrate that the rams were capable of discriminating between the odours. The aversive effect was only seen when S+ was changed from frozen/thawed non-oestrous urine odour (trial 2) to fresh non-oestrous urine odour (trial 3). High error scores on day 1 do not show decreased discrimination but rather the tendency to press S-, rather than S+, despite not being rewarded.

Experiment 2: Effect of freezing/thawing non-oestrous urine.

The rams showed individual variation in their ability to discriminate between the odours of fresh and frozen/thawed non-oestrous urine samples from the same ewe. Three of the four rams could clearly discriminate between the urine odours from ewe 181 (2a, figure 11) whereas two of the four could discriminate between the urine odours from ewe 180 (2b, figure 11).



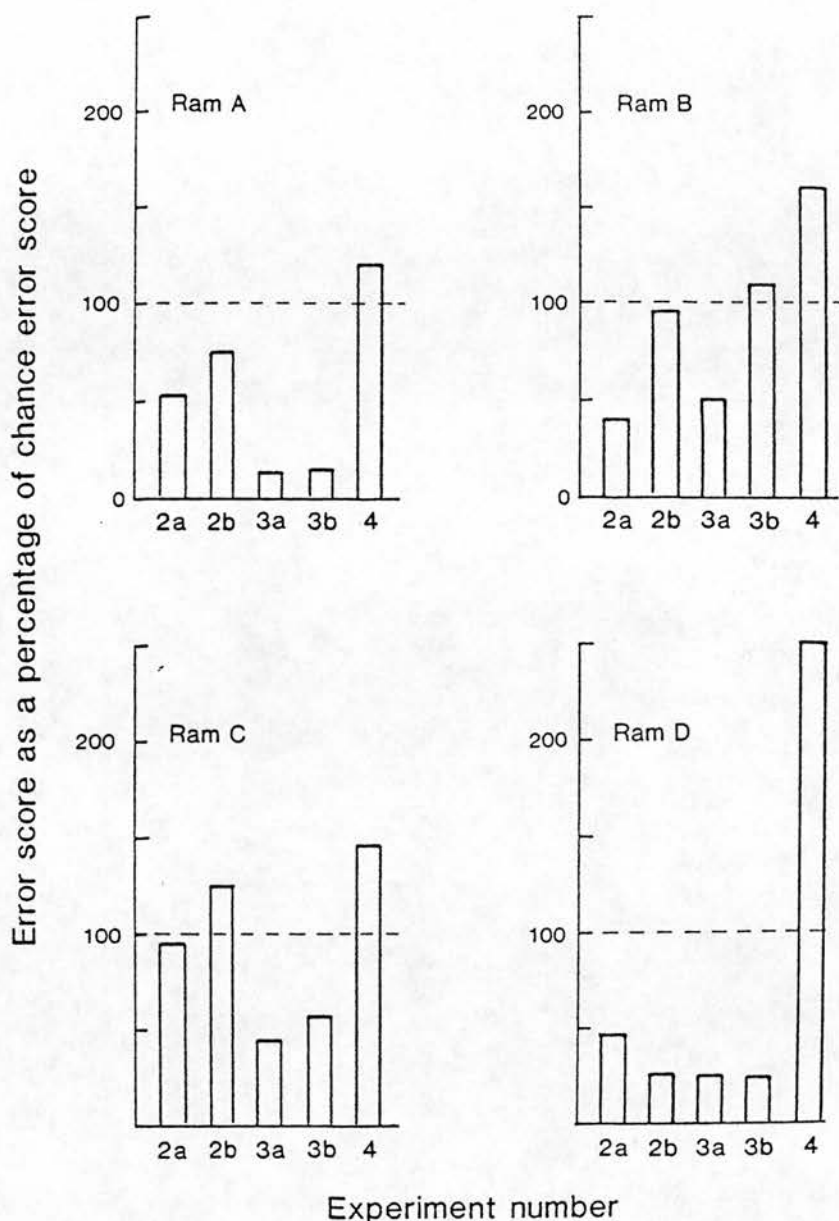


Figure 11. Error scores achieved by four rams discriminating between the odours of fresh and defrosted oestrous (experiment 3) and non-oestrous (experiment 2) ewe urine collected from ewe 181 (a) and ewe 180 (b). Control experiment (experiment 4) - same odour delivered to both odour nozzles.  
 - - - = Chance.

### Experiment 3: Effect of freezing thawing oestrous urine.

All four rams could discriminate between the odours of fresh and frozen/thawed oestrous urine odour from ewe 181 (3a, figure 11). Three of the four rams could discriminate between these odours from ewe 180 (3b, figure 11).

### Experiment 4: Control - same odour both sides.

Rams were unable to select the switch associated with the food reward using confounding stimuli (figure 11). The validity of the apparatus and experimental procedure in providing a simultaneous olfactory discrimination test was therefore confirmed.

### Transfer of discriminatory ability.

If the basis for the discrimination between urine odours was common to all oestrous cycles of all ewes tested, then rams would be able to transfer their ability to discriminate to trials with novel oestrous and non-oestrous urine odours. By testing on successive days with urine samples from different ewes and from different oestrous cycles, any conspecific differences in urine odour or any differences due to particular oestrous cycles could be eliminated. The only consistent feature of S+ and S- was the oestrous or non-oestrous state of the donor ewe.

Consistently low error scores are not sufficient to imply positive transfer. Low error scores could be achieved if a "learning set" had been established (Harlow 1949; Mackintosh 1974). To guard against this

possibility an analysis of the rams first choices was made for each of the odour combinations in Experiment 1. The switches sniffed by the rams before starting to press were recorded. The results of this analysis are shown in Table 2.

The probability of the observed number of correct first choices, or better, being achieved by chance was calculated using the binomial distribution. Three rams showed positive transfer to previously untested odour combinations ( $P < 0.01$ ) and one achieved 12 out of 17 correct first choices ( $P > 0.05$ ).

Rams were correct on 57 out of 68 occasions (84%). On 48 of these 57 occasions the rams sniffed both odours and on 9 occasions they sniffed only the S+ odour before starting to press switches. For days 2 to 6, when frozen/thawed non-oestrous urine odour was S+, the rams made 100% correct first choices. For days 9 to 11, when fresh non-oestrous urine odour was S+, their performance fell to 66% correct. For days 12 to 22, when the former S+ was used again the rams achieved 87% correct first choices. These results indicate that rams were capable of positive transfer when given previously untested odours.

Curiously the rams were never seen to flehmen in the crate and yet non-oestrous urine is known to stimulate the flehmen response in the field.

TABLE 2. CORRECT AND INCORRECT FIRST CHOICES MADE BY RAMS IN OLFACTORY DISCRIMINATION EXPERIMENTS USING OESTROUS AND NON-OESTROUS EWE URINE. URINE WAS FRESH OR FROZEN AND THAWED AS SHOWN.

TRIAL NUMBER	1		2		3		2		1		2		TOTAL NUMBER CORRECT					
	1	2	3	4	5	6	9	10	11	12	13	14		18	19	20	21	22
DAY	1	2	3	4	5	6	9	10	11	12	13	14	18	19	20	21	22	
RAM A	▽	●	●	▼	●	●	●	○	●	●	●	●	●	●	●	●	●	15 (P<0.01)
RAM B	▽	●	●	●	●	●	○	○	●	●	●	○	●	●	▽	●	●	12 (N.S.)
RAM C	●	●	●	▼	●	●	▼	▼	○	●	●	○	▼	●	▼	▽	●	14 (P<0.01)
RAM D	▽	●	●	▼	●	●	●	▼	●	●	▼	●	●	●	●	●	●	16 (P<0.001)

- ▽ Ram sniffed S+, then chose correctly.
- Ram sniffed S+ and S-, then chose correctly.
- ▽ Ram sniffed S-, then chose incorrectly.
- Ram sniffed S+ and S-, then chose incorrectly.

- TRIAL 1: S+ frozen/thawed non-oestrous urine odour.  
S- fresh oestrous urine odour.
- TRIAL 2: S+ frozen/thawed non-oestrous urine odour.  
S- frozen/thawed oestrous urine odour.
- TRIAL 3: S+ fresh non-oestrous urine odour.  
S- frozen/thawed oestrous urine odour.

## DISCUSSION.

These results demonstrate that rams can detect an odorous difference between oestrous and non-oestrous ewe urine, when it has been frozen and thawed. Having learnt to respond to the difference between the odours, three of the four rams were able to transfer this ability and discriminate between similarly prepared, previously untested, urine odour combinations. It was therefore possible to train rams to identify receptive ewes by the odour of their defrosted urine. It has been shown previously that rams can discriminate between urine odours from conspecific ewes without reference to the state of sexual receptivity (Baldwin & Meese 1977). Care was taken in these experiments to change the donor ewe regularly so as to avoid the rams becoming trained to recognise the odours of an individual ewe.

The duration of freezing the urine did not seem to affect the rams ability to discriminate between oestrous and non-oestrous urine odour. Defrosted urine samples were used which had been frozen for periods ranging from 30 minutes to 250 days. Rams could also discriminate between oestrous and non-oestrous urine odours if one of the pair was fresh and the other was frozen and thawed. Furthermore the results show that fresh non-oestrous urine odour was aversive when first presented. The rams did not perform better when S- was changed to fresh oestrous urine odour from frozen and thawed oestrous urine odour (when trial 2 was changed back to trial 1)



suggesting that rams do not avoid novelty per se or avoid fresh urine odour when given the alternative of pressing switches associated with frozen and thawed urine odour. The rams quickly acquired the ability to overcome the aversive effects of fresh non-oestrous urine odour and no such effects were evident on the second or third day of the trial.

Unfortunately these results also demonstrate that freezing and thawing both oestrous and non-oestrous ewe urine changes their odours as perceived by most of the rams. This conclusion contrasts with reports that male guinea-pigs are unable to discriminate between fresh and defrosted urine (Beauchamp et al. 1982). It is not clear which urine fractions are affected by freezing: volatiles may be removed or added, or the concentrations of existing volatiles may be altered. Indeed the odour of defrosted ewe urine smells quite different from the smell of fresh ewe urine even to the human subject. It is concluded that the odour of ewe urine which has been stored by freezing, and is subsequently thawed, is therefore artifactual. This finding establishes the need to use fresh ewe urine in odour experiments if the results are to be relevant to the understanding of ovine odour communications in the field. Little work has been reported for other species but in the light of these findings it would seem prudent to refrain from using frozen and thawed urine in odour experiments until it is

established that the odour of the sample is not affected by the storage processes. To overcome this difficulty it is necessary either to devise a means of storing urine which does not affect its odour, or to design a series of olfactory discrimination experiments using fresh oestrous and non-oestrous urine from different ewes and different oestrous cycles, such that the only common variable between the two categories of urine is the receptive state of the donor ewe. The result would then indicate whether rams can detect fresh urine volatiles related to the two different stages of the ewes sexual cycle. This course of action was followed (Chapter 4).

The absence of a flehmen response by the rams to both defrosted and fresh urine under the trial conditions is intriguing. The flehmen response was clearly not necessary for the rams to discriminate between the odours of oestrous and non-oestrous urine. The absence of flehmen may be due to presentation of urine odours out of context i.e. the rewards in the crate were edible and not sexual. Ewe urine volatiles, as presented in this work did not initiate flehmen by rams.

#### CHAPTER 4. RAMS' MEMORY OF EWE URINE ODOURS BETWEEN BREEDING SEASONS AS ASSESSED BY REVERSAL LEARNING.

##### Introduction.

In Chapter 3 the artifactual nature of defrosted ewe urine odour was established. Because of difficulties in finding a method of storage which did not affect the odour of ewe urine, odour experiments were discontinued until November 1988, when fresh oestrous urine again became available. This provided the opportunity to determine if rams could remember the odours after this interval and to investigate long term memory of odours in rams.

##### Experiments and Procedure.

The four rams were reintroduced to olfactory discrimination experiments using the same apparatus as before (Chapter 2). The odour stimuli were frozen/thawed oestrous and non-oestrous ewe urine odours. The urine had been tested near the end of the previous breeding season and was stored frozen in the interim.

To assess the rams ability to remember two odours, the relevance of the odours to the food reward was reversed. Frozen/thawed oestrous urine odour was therefore the new S+ (previously S-), and frozen/thawed non-oestrous urine odour was the new S- (previously S+). If the rams retained any memory of the significance of the odours then very high initial error scores would be expected as rams would press on the switch associated

with the new S- because it had previously been associated with the food reward.

Reversing the significance of oestrous and non-oestrous urine odours was necessary for 2 further reasons:

1. To determine if similar levels of discrimination could be achieved with the reward values of the odours reversed.

2. To retrain the rams to accept frozen/thawed oestrous urine odour as S+. This was necessary to allow discrimination experiments between frozen/thawed and fresh oestrous urine odour to investigate the effects of freezing and thawing on oestrous urine odour (Chapter 3).

It was not known if the rams would remember how to use the apparatus after such a long period of time. The assessment of the ram's memory of odours depended on the animals being able to remember how to use the apparatus.

Ten consecutive daily olfactory discriminations were completed by the rams using the following odour stimuli:

S+ frozen/thawed oestrous urine odour (ewe 182, sample date 15/2/88).

S- frozen/thawed non-oestrous urine odour (ewe 182, sample date 5/2/88)

The two odours had previously been tested on 18/2/88 and the reversal experiments were started on

5/11/88. The period between the two experiments using the same odour stimuli (with their significance reversed) was 259 days. The rams had completed other discriminations using frozen/thawed urine at the end of the previous season. The period for which the rams did not use the apparatus was 247 days. Distribution of error scores over the 50 choices were recorded daily for each ram.

### RESULTS.

All rams entered the crate, sniffed both odours and started pressing switches. There was no indication that any ram could not remember how to use the apparatus.

Figure 12 shows the error scores (learning curves) for each ram learning the reversal. The 2 Suffolk rams scored below chance levels on day 2 (Ram D) and day 3 (Ram A) and thereafter could discriminate consistently. The 2 Cheviot rams were slower to learn the reversal. Ram C did not perform below chance levels consistently until day 7, and Ram B did not achieve this until day 8. After 10 days all rams were discriminating between odours successfully. The final levels of accuracy attained here, with S+ and S- reversed, were not significantly different from those in the earlier work (students t-test).



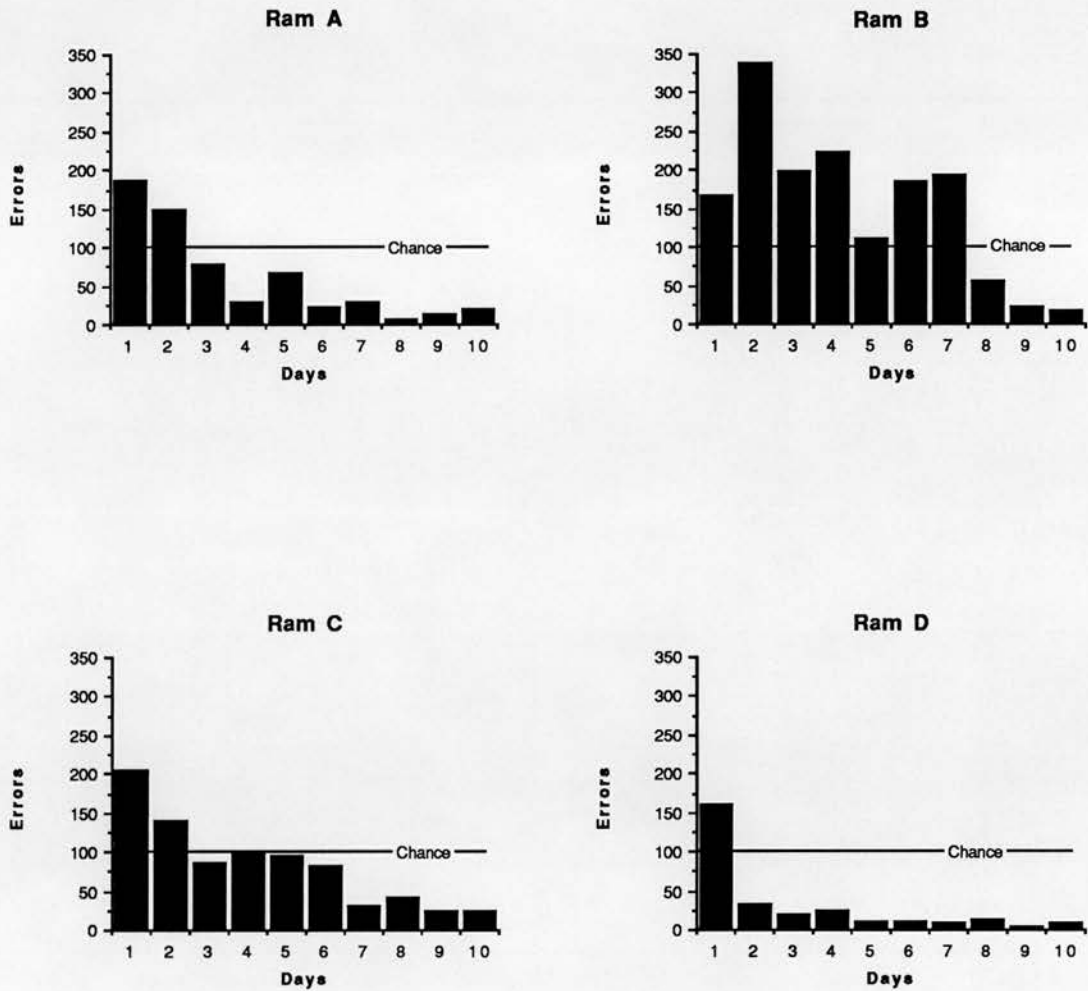


Figure 12. Error scores for four rams learning the reversal (S+ = defrosted oestrous urine, S- = defrosted non-oestrous urine) of a previously learnt discrimination (S+ = defrosted non-oestrous urine, S- = defrosted oestrous urine). Learning curves show rate of learning the new task.

S+ Non-oestrous urine odour. S- Oestrous urine odour.

Errors.

	Ram A	22	
18/2/88	Ram B	33	Mean = 24.5
	Ram C	27	
	Ram D	16	

S+ Oestrous urine odour. S- Non-oestrous urine odour.

Errors.

	Ram A	13	
5/11/88	Ram B	32	Mean = 21.75
	Ram C	31	
	Ram D	9	

On day 1 of the reversal experiment rams A, B, C, and D scored 188, 167, 204 and 160 errors respectively. To complete the 50 choices the minimum number of presses required at FR4 is 200. Therefore over the 50 choices the rams did not press S- more than S+ providing no indication of memory of the relationship between odours and food rewards between breeding seasons.

There was an even distribution of errors over the 50 choices on day 1.

DISCUSSION.

Whilst rams began using the apparatus immediately upon its reintroduction, there was no indication that they remembered the significances of the test odours from the previous season.

It appears that rams learnt the olfactory

discrimination as a novel test. Unfortunately it was not possible to compare the learning curves in figure 12 with the equivalent curves for the original discrimination. Learning curves for the earlier experiment could not be constructed because S+ in that case had already been presented in training and rams therefore had prior experience of it.

Similar reversal learning experiments have been used in rats to investigate odour discriminations and memory. It has been shown that rats can remember 2 odours tested by reversal 4 weeks later (Staubli, Fraser, Faraday & Lynch 1987). Staubli, Schottler & Nejat-Bina (1987) demonstrated that rats can remember 2 odours after 3 weeks even when other olfactory discriminations were conducted in the interim.

It is unlikely that rams forget sexually significant odours between breeding seasons, however the association of specified odours with food rewards is not apparently maintained.

## CHAPTER 5. DISCRIMINATION BETWEEN THE ODOURS OF FRESH OESTROUS AND FRESH NON-OESTROUS EWE URINE BY RAMS.

### Introduction.

The aim of these experiments was to determine if rams could discriminate between volatiles from fresh oestrous and fresh non-oestrous ewe urine. Previous work (Chapter 3) demonstrated that rams can discriminate between volatiles from oestrous and non-oestrous ewe urine which has been stored by freezing. However the discovery that freezing and thawing changed the odour of the urine, as perceived by rams, limited the conclusions that could be drawn about the role of such volatiles in the field. In this work fresh oestrous and non-oestrous urine odours, from novel combinations of ewes and oestrous cycles, were presented to rams each day. The ability of rams to discriminate between the odours was investigated. The ability of rams to choose fresh oestrous urine odour (S+) instead of fresh non-oestrous urine odour (S-) in the first choice of each experiment was also investigated. If rams are able to choose the oestrous sample when presented with a series of previously untested pairs of odours then the presence of an "oestrus-indicating" odour common to different ewes and different oestrous cycles is indicated.

### EXPERIMENTS.

#### Olfactory discriminations.

Twenty two olfactory discrimination trials were

completed by the rams. On each test day the rams were presented with one fresh oestrous (S+) and one fresh non-oestrous (S-) urine sample from different combinations of ewes and oestrous cycles (Table 3). The only consistent feature of the positive discriminative stimulus odour was the oestrous state of the donor ewe and the only consistent feature of the negative discriminative stimulus was that the donor ewe was in the luteal phase of the cycle (days 7 to 11). This ensured that rams did not become trained to the urine of any individual ewe or to any particular day. It was not always the case that when one ewe was in oestrus another was on day 9 of the oestrous cycle. The non-oestrous donor ewe was therefore either on day 9 or the nearest day to this (Table 3).

#### First choice analysis.

To assess the rams' ability to choose "novel" oestrous urine odours in preference to non-oestrous urine odours (for a food reward) it was necessary to analyse the results of the first choice which rams made on each test day. Consistently low daily error scores are not in themselves enough to imply positive transfer of discriminative behaviour to previously untested odours. If a "learning set" has been established (Harlow 1949; Mackintosh 1974) the subject is able to determine which of the two stimuli is associated with the food reward after the first choice has been made, regardless of whether it was correct or incorrect. Such "within



TABLE 3 Urine donors in 22 olfactory discrimination trials. The day of the oestrous cycle on which the "non-oestrous" sample was taken is shown. All oestrous samples are from days of "standing oestrus". S+ is the positive discriminative stimulus. S- is the negative discriminative stimulus.

TRIAL NUMBER	OESTRUS URINE DONOR (S+)	NON-OESTROUS URINE DONOR (S-)	DAY OF CYCLE (S-)
1	198	180	10
2	180	198	7
3	180	198	8
4	197	198	9
5	181	199	9
6	198	197	8
7	198	180	11
8	199	181	10
9	180	198	6
10	180	198	7
11	197	198	9
12	198	197	9
13	198	197	10
14	180	181	10
15	180	198	7
16	199	198	8
17	197	198	9
18	198	197	10
19	198	181	10
20	180	181	11
21	197	198	9
22	181	180	8

trial" learning has not previously been demonstrated in sheep, but nevertheless the first choice only, in each experiment, was assumed to be a valid measurement of positive transfer to previously untested odour combinations. For each initial choice of the 50 choices the rams made in each experiment, the rams were scored "correct" or "incorrect" according to whether the switch associated with S+ or S- first received 4 presses.

Control experiments were conducted. The same oestrous urine odour was delivered through both odour nozzles simultaneously. The rams were unable to select the switch which would provide the food reward when pressed and were therefore not using confounding stimuli to perform the discriminations.

#### RESULTS.

The daily error scores for each ram are shown in figure 13. All rams could discriminate between oestrous and non-oestrous ewe urine odours on all occasions. Rams A and D (Suffolk rams) achieved better daily error scores than rams B and C (Cheviot rams).

Table 4 shows the results of the rams first choice on each day. For the first 12 days the rams chose the "novel" oestrous urine odour on 27 out of 48 occasions (N.S.). For the last 10 days the rams correctly chose the oestrous urine odour on 37 out of 40 occasions ( $P < 0.001$ ). The possibility of the number of correct choices being achieved by chance was calculated using

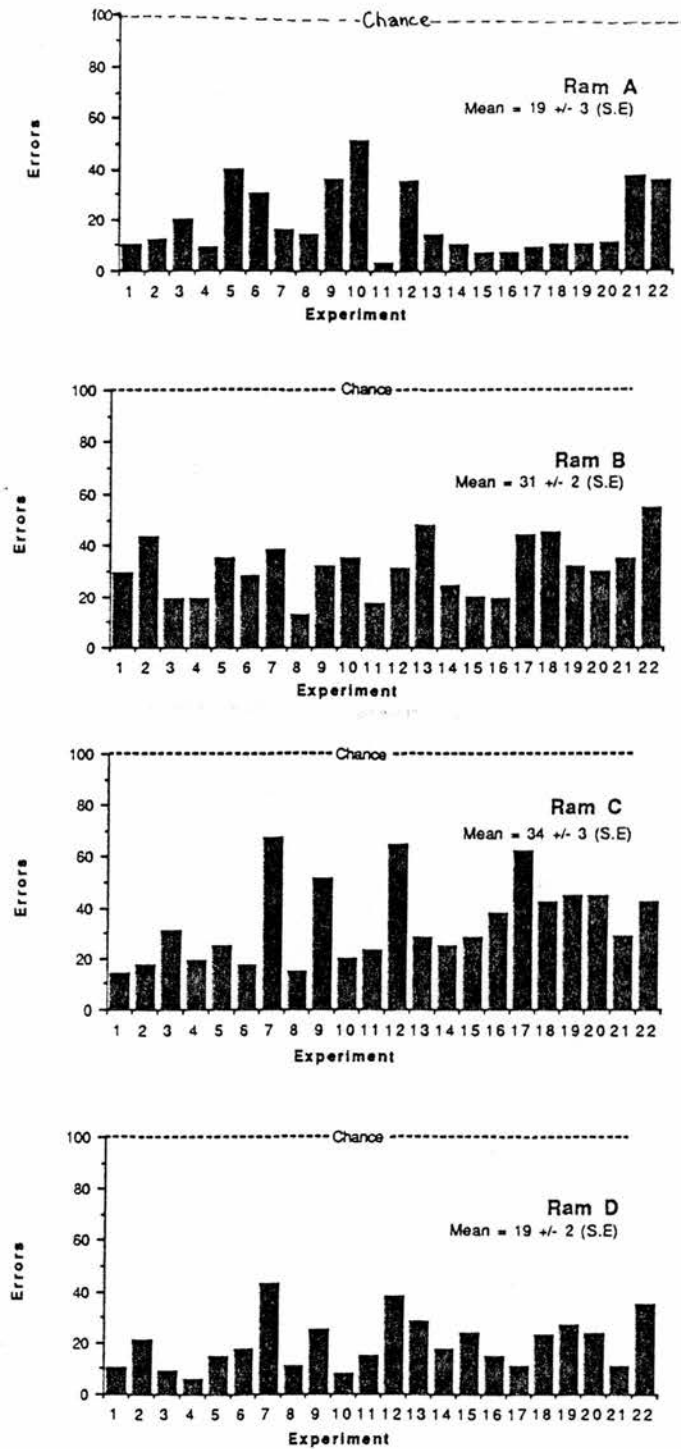


Figure 13. Daily error scores for four rams during 22 olfactory discrimination experiments using fresh oestrous and non-oestrous ewe urine odours from different ewes and different oestrous cycles.  
- - - = Chance error level.

TABLE 4. Correct and incorrect first choices made by rams in 22 olfactory discrimination trials using previously untested oestrous and non-oestrous ewe urine odours.

	TRIAL																						P value last 10 trials
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
RAM A	○	●	○	○	●	●	○	○	●	●	○	●	○	○	○	○	○	○	○	○	○	○	P<0.01
RAM B	●	○	●	○	●	○	●	○	●	○	○	○	○	○	○	○	●	○	○	○	○	○	P<0.01
RAM C	●	●	●	○	●	○	●	○	○	○	○	●	○	○	○	○	○	●	○	○	○	○	P<0.01
RAM D	○	○	○	○	●	●	○	○	●	○	○	●	○	○	●	○	○	○	○	○	○	○	P<0.01

● = incorrect choice

○ = correct choice

Rams A and D - Suffolk

Rams B and C - Cheviot

the binomial distribution. The performances of all rams over the final 10 trials were statistically significant.

#### DISCUSSION.

These results demonstrate for the first time that rams can discriminate between fresh urine odours from oestrous and non-oestrous ewes. The basis of this difference is not yet established and preliminary chromatographic assays have been inconclusive (Jubilan 1983; Bland, K.P., Jubilan, B.M., Lang, C.W., & Nizamlioglu M., - personal communication). It may be due to volatiles which occur at different stages of the oestrous cycle or alternatively it may be due to varying concentrations of urine volatiles which correlate with the degree of sexual receptivity. By using the methods employed in this work, progress can be made in determining the nature and source of the odorous difference (and its means of detection by the ram) without the immediate need to know its chemical basis, although this will be necessary to allow a full understanding of this area of odour communications.

It is demonstrated here that the difference between the odours of fresh oestrous and non-oestrous ewe urine is consistent for different ewes and for different oestrous cycles. All rams could detect the difference. The rams learnt to respond to the difference over the first 12 experiments (when the results of their first choices were not significantly different from random choice). During this time they would have had to learn



to ignore other odour cues which could have been present and respond exclusively to the odour cue which signified whether the urine odour was oestrous or non-oestrous in order to obtain the food reward most efficiently. This was achieved, as results of the final 10 trials demonstrate.

These findings must be interpreted in terms of what is known about sheep courtship behaviour. Non-oestrous ewes characteristically urinate in response to the approach of the ram (Geist 1971; Bland & Jubilan 1987). The rams attentions then focus on the voided urine and the flehmen response is commonly observed (Banks 1964). The ewe usually leaves the scene during these activities. Conversely, oestrous ewes do not typically urinate to the rams approach (Geist 1971; Bland & Jubilan 1987). It may appear that there is no need for an olfactory difference between urine samples, because the sight of a ewe urinating when approached should tell the ram that she is unreceptive without the need for lengthy olfactory investigations involving flehmen behaviour. However the ram does spend a considerable period of time investigating non-oestrous ewe urine which suggests that this is biologically advantageous in some way. These experiments establish that urine from sexually receptive ewes smells different from the urine of sexually unreceptive ewes. It therefore seems likely that olfactory investigation of ewe urine by rams is

intended to monitor the change of odour from that which is characteristic of sexually non-receptive ewes to that which is characteristic of sexually receptive ewes. If this change occurred a small time before "standing oestrous" then its detection by the ram would enable him to predict impending oestrus. It would be advantageous for the non-oestrous ewe to urinate frequently when the ram approached so as to increase the likelihood of the ram recognising her impending change of sexual state. Future work is required to determine at what stage of the oestrous cycle the odour of non-oestrous urine changes to that characteristic of oestrous ewes. By examining the rams' ability to discriminate between oestrous urine odours and odours of urine from days of the oestrous cycle preceding oestrus, it should be possible to resolve this question.

The olfactory discrimination technique employed establishes that rams can discriminate between odours, but not that they actually use this ability in the field. It is possible that rams were only discriminating between urine odours because they had been trained to do so. This seems unlikely as rams would be expected to use all the sensory information available to them in order to detect receptive females.

The levels of ability achieved by rams here was very similar to those achieved previously, in a different season, using frozen urine samples (Chapter 3). It is interesting that Suffolk rams seem to perform

better than Cheviot rams. Examination of the videotape (Appendix B) reveals that the Suffolk rams press switches by biting them with their lower jaws, whereas the Cheviot rams press by making sideways movements of the head. The technique of the Suffolk rams may allow them to discriminate odours more accurately because their nostrils are held over the odourous airstreams when pressing switches.

Rams did not flehmen when performing their olfactory discriminations. This was also found during previous work using stored urine samples (Chapter 3). Thus flehmen is not necessary for the ram to distinguish between oestrous and non-oestrous urine volatiles. Future experiments should be directed towards determining the chemical basis of the odour difference demonstrated and the location of chemoreceptive fields which mediate its detection by the ram.

CHAPTER 6. THE INVOLVEMENT OF INTRANASAL AND VOMERONASAL  
CHEMORECEPTION IN THE DISCRIMINATION OF OESTROUS AND  
NON-OESTROUS EWE URINE ODOURS BY RAMS.

INTRODUCTION.

It was established that fresh oestrous ewe urine has a different odour to fresh non-oestrous ewe urine, as perceived by rams (Chapter 5). However the location of chemoreceptors which mediate this discrimination remained unclear. In the mammalian nose there are five neural sensory systems which might be involved (Tucker 1971; Graziadei 1977; Keverne, Murphy, Silver, Wysocki & Meredith 1986):

1. The olfactory neuroepithelium.
2. The vomeronasal organ (VNO).
3. The septal organ of Maser.
4. Nervus Terminalis nerve endings.
5. Sensory endings of the trigeminal nerve.

The aim of this work was to determine the respective roles of intranasal and vomeronasal chemoreception in this olfactory discrimination.

Whereas the main olfactory epithelium is normally stimulated by volatile stimuli, the vomeronasal neuroepithelium might be stimulated by volatile or non-volatile molecules. As Stevens (1984) has stated, many authors believe the VNO to be involved primarily with the chemoreception of non-volatile stimuli (Altieri & Müller-Schwarze 1980; Beauchamp et al. 1980; Jacobs, Sis, Chenoweth, Klemm, Sherry & Coppock 1980; Johns 1980).

However, as Meredith (1982) has pointed out, there is no direct evidence that non-volatile substances can stimulate the vomeronasal receptors but such evidence does exist for volatile odours (Tucker 1963; Müller 1971; Tucker 1971).

Sensory deprivation techniques have been used by other workers to induce behavioural deficits and investigate the location of chemoreceptive fields. Temporary olfactory deficits in rams have been reported following intranasal irrigation with 2% xylocaine hydrochloride solution (Banks, Bishop & Norton 1963; Alberts 1974). A polythene tube, with pin holes in the sides, was inserted into the nose and a 5ml syringe was attached. Pressure on the syringe produced a fine spray of anaesthetic agent. The deficit was verified by testing the withdrawal reaction of blindfolded rams when ether soaked cotton balls were held 6" from the nose.

A similar technique has been described in ewes (Chapple 1984). 0.5ml of lignocaine (10mg) was applied as a fine spray to each nostril, from the external nares to approximately 8cm along the nasal passage. The deficit was verified with a head withdrawal test using cotton wool soaked in glacial acetic acid held 6cm from the animals nose. This technique has recently been adapted for use in lambs (Vince, Lynch, Mottershead, Green & Elwin 1987) using 10% lignocaine hydrochloride spray.



There are no previous reports of methods for reversibly depriving sheep of their VNOs. Laboratory rodents are usually deprived of their VNO by irreversible lesioning or surgical excision (hamsters - Meredith 1986; Clancy, <sup>Macrides,</sup> et al. 1984; mice - Bellringer et al. 1980; Wysocki, Nyby, Whitney, Beauchamp & Katz 1982; Lomas & Keverne 1982; guinea pigs - Beauchamp et al. 1982, Beauchamp, Wysocki & Wellington 1985; rats - Fleming et al. 1979 and voles - Lepri & Wysocki 1987)

Previous workers have made unsuccessful attempts to obstruct stimulus access to the VNO of cattle (Klemm et al. 1984) and cats (Verberne 1976) by blocking the nasopalatine canals. Ladewig et al. (1980) surgically obstructed the oral end of the NPCs of goats but this did not block the VN duct as the VN duct opens into the nasal cavity in goats (Melese d'Hospital & Hart 1985; Hart 1983). The first successful method for reversible deprivation of the VNO in sheep is described in this work.

#### METHODS.

Three sensory deprivation techniques were employed:

1. Topical intranasal local anaesthesia.
2. Reversible obstruction of the nasopalatine canals and vomeronasal ducts, thus preventing stimulus access to the vomeronasal organ.
3. Intranasal irrigation with 1% zinc sulphate and 4% Procaine solution.

## Sensory deprivation techniques.

### 1. Topical intranasal local anaesthesia.

Attempts were made to induce temporary olfactory deficits using local anaesthetic applications as described above. None was successful. After each attempt the behavioural verification test of Poindron (1974) was used to test for an olfactory deficit: rams were offered oats in a bucket containing a fine mesh floor with dog faeces underneath. The sheep was judged to have an olfactory deficit if it ate from the contaminated bucket for more than 5 seconds. Control experiments were conducted using similar buckets with no faecal contamination.

Various doses (10mg to 200 mg per nostril, the maximum advised human dose is 200mg) of Xylocaine spray (Astra pharmaceuticals Ltd, England) were applied in metered 10mg spray doses into each nostril. No olfactory deficit could be demonstrated. An aerosol apparatus was constructed which produced a spray of xylocaine hydrochloride solution from the nozzle of a 0.5cm diameter tube which could be inserted to the back of the rams nose. The nozzle tip was angled dorsally to direct the spray to the olfactory epithelium. All attempts to impair olfaction by these methods were unsuccessful.

Substitution of 5% procaine hydrochloride solution for the xylocaine spray was similarly unproductive. The procaine was administered as a spray or as drops at doses of up to 5ml per nostril. The posture of the rams

was varied prior to administration of the drug to try to ensure sufficient contact between the drug and the olfactory epithelium but no method was successful in producing a deficit.

## 2.Obstruction of the nasopalatine canal and vomeronasal duct.

It was not considered feasible to remove the VNO from rams without causing considerable disruption to surrounding tissues. The specificity of any deficits would be doubtful. A reversible technique was favoured to allow trained rams to return to discrimination experiments.

### Anatomy.

The anterior nasal cavities and hard palates of 6 adult rams were dissected and the relationships of the nasopalatine canals (NPCs), vomeronasal ducts (VN ducts), and the nasal cavities were established. The nasopalatine canals of the sheep (Goodall 1912; May 1964) are bilaterally symmetrical ducts which allow communication between the mouth and the nose. The oral opening of each canal is located either side of the incisive papilla (figure 14). The canals are first directed caudolaterally and then dorsocaudally to open into the anterior nasal cavity. The opening into the nasal cavity is wedge-shaped, being much wider at the nasal end than the buccal end (figure 15). The entrance to the VNO is via a slit-shaped opening into the VN duct



Figure 14. Hard palate of ram showing oral openings of nasopalatine canals.

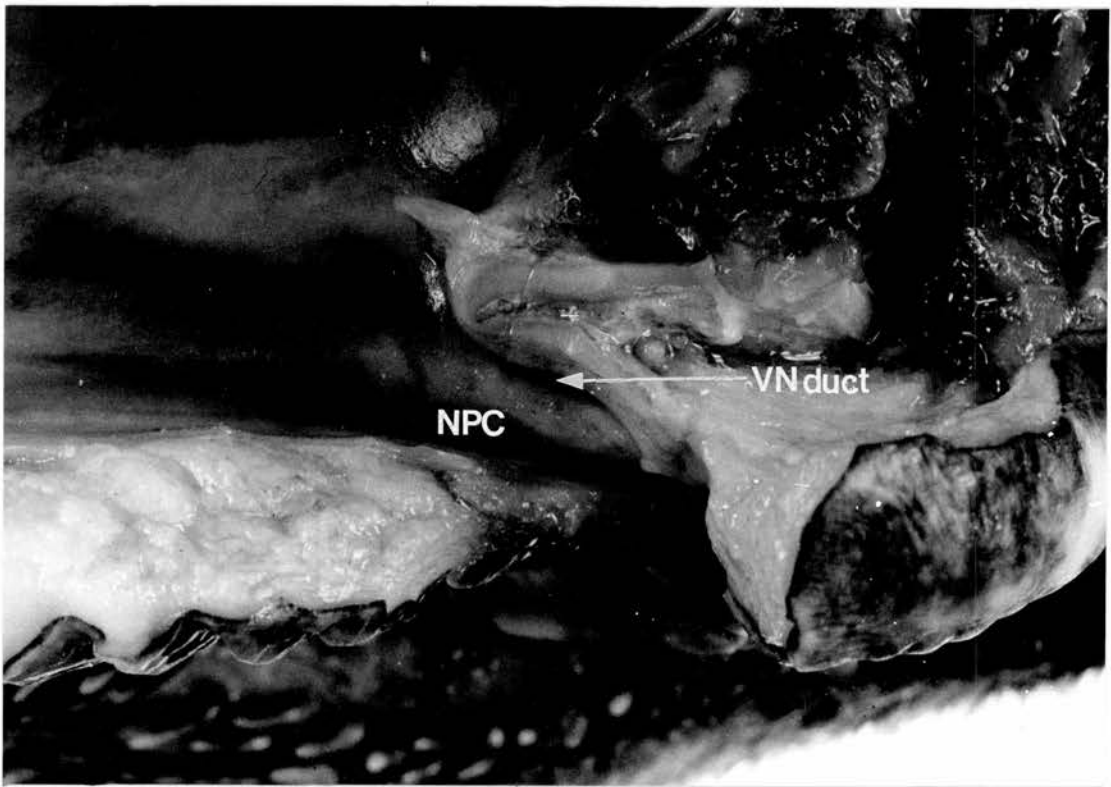
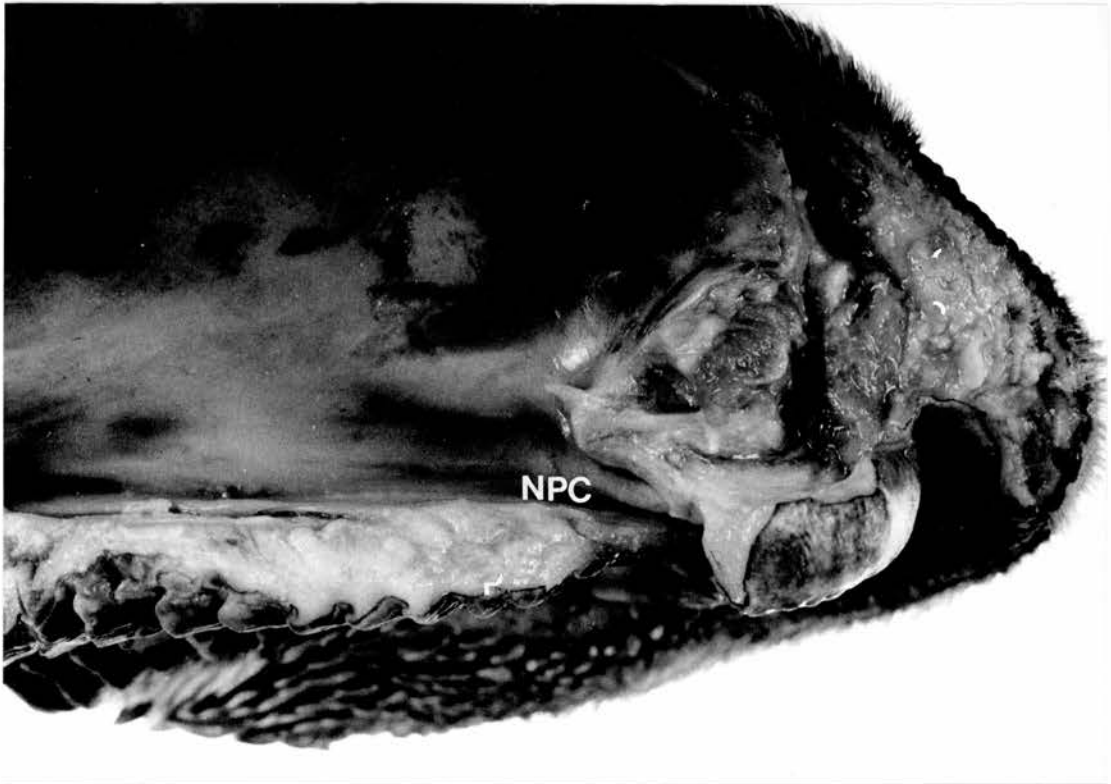


Figure 15. Right nasal cavity of a ram showing anatomical relationships between nasal cavity, nasopalatine canal (NPC) and vomeronasal duct opening (VN duct)



which is located mid-way along the length of the canal on its medial wall (figure 15). The slit-shape is orientated rostrocaudally. The anatomy of these structures is similar to that of the bovine (Jacobs et al. 1981) but different to that of the caprine where the VN duct opens directly into the nasal cavity (Hart 1983).

#### Techniques used.

The nasopalatine canals of rams were physically obstructed using purpose-made bungs. Three brass molds were used to manufacture different sizes of bungs (figure 16). The bungs blocked the nasopalatine canals, and entrances to the VN ducts, of all dissection specimens.

Liquid vinyl polysiloxane (Reposil HF, De Trey Ltd) was poured into molds around a stainless steel wire (30 gauge). The end of the wire contained in the mold was soldered to a small flat washer so that the wire gripped the bung and could be used to manipulate it into position in the animal. The wire was covered with the smallest diameter polyethylene catheter that would slide over it, to provide a protective surface.

Anaesthesia was induced in Rams C and D using a 4% Fluothane (Halothane) /Nitrous oxide mixture in oxygen, administered by facemask. Rams were intubated and anaesthesia maintained with 2% Fluothane in oxygen. A stainless steel "guide-wire" , with a loop on one end, was introduced into the buccal end of the left NPC and

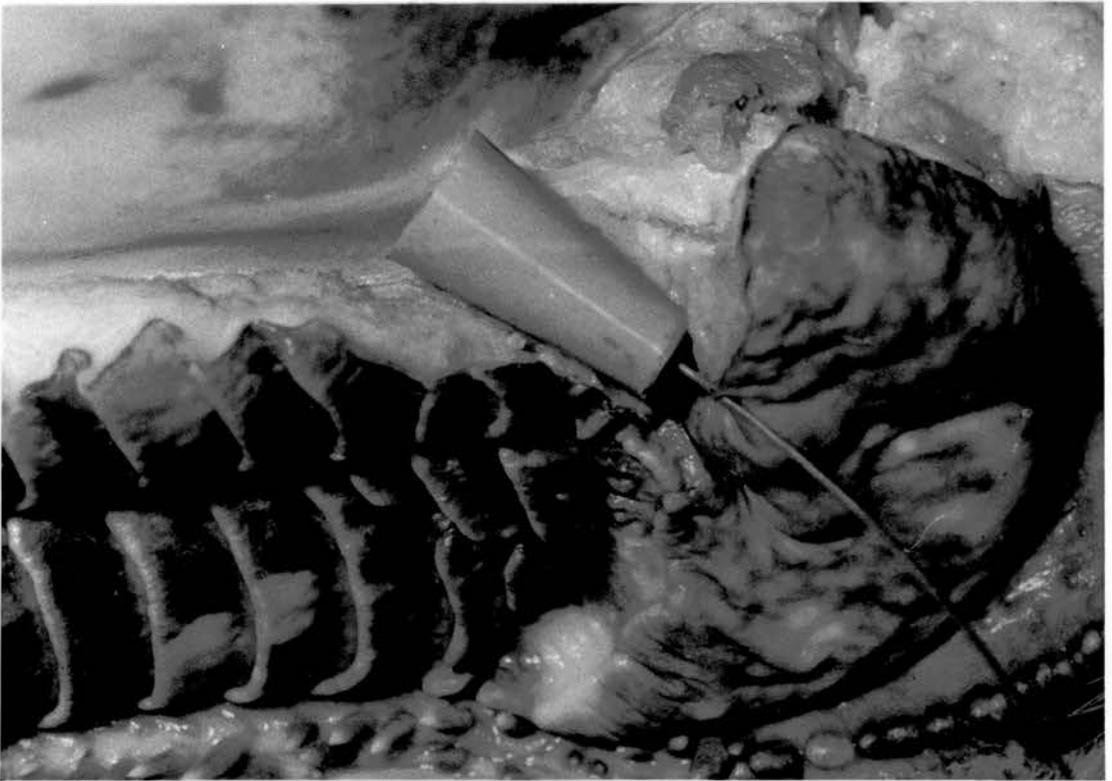
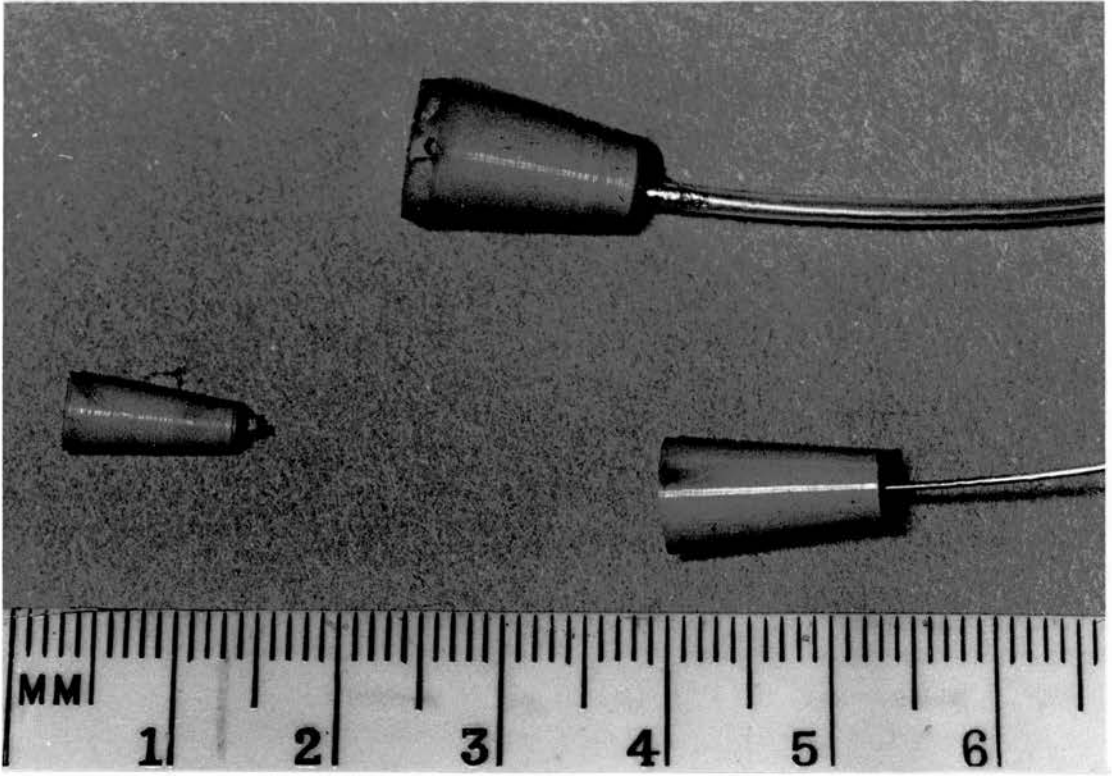


Figure 16. Bungs used to block the nasopalatine canals.

advanced until the loop could be seen in the nose. The loop was grasped and withdrawn through the external nares. The "bung-wire" was attached to the loop and the guide-wire was gently withdrawn through the buccal opening of the NPC. When the bung-wire appeared in the mouth the guide-wire was detached and discarded. By gentle pulling on the bung-wire the bung was wedged firmly into a position which occluded the nasopalatine canal and the associated vomeronasal duct. The procedure was repeated on the right side and the two bungs were anchored by knotting the two bung-wires across the roof of the mouth. The knot was tied in such a way as to leave no protruding edges which could harm the rams. A minimum period of 48 hours was allowed before the rams were reintroduced to pressing switches. Rams fitted with bungs did not appear to behave differently to intact rams.

### 3.Zinc sulphate irrigation of the nasal cavities.

The method adopted for  $\text{ZnSO}_4$  irrigation of the nasal cavities was originally described by Poindron (1974). Irrigation was conducted whilst the NPC bungs were still in position to ensure that the VNO was protected from the irrigating fluid.

Rams were injected intramuscularly with 3mg dexamethasone acetate (Azium injection, Gist-Brocades) 4 hours prior to surgery and 100mg promethazine (Phenergan, May and Baker) immediately prior to

irrigation, to reduce the effects of shock associated with the procedure (P.Poindron - personal communication). Anaesthesia was induced and maintained as before. Rams were placed in dorsal recumbency with their necks extended. The olfactory epithelium was the lowest point of the nasal cavity. The cuff on the endotracheal tube was inflated to ensure that irrigating fluid was not aspirated. Both nasal cavities were irrigated with 1%  $\text{ZnSO}_4$  and 4% procaine hydrochloride solution. The solution was left in the nose for 3 minutes (Ram D) and 15 minutes (Ram C) respectively. Ram C suffered a period of apnoea at the time of irrigation. Periods of apnoea are not uncommon with this technique (P.Poindron - personal communication) and may be due a sudden excitation of the trigeminal input. During this episode the rams heart rate remained steady and recovery was uneventful. The  $\text{ZnSO}_4$  solution was removed by altering the position of the head to allow drainage.

Donor ewes.

Fresh oestrous and non-oestrous urine samples were collected from ewes as previously described (Chapter 5). Because of the small number of urine donors, "day 9 urine" was not always available when ewes were in oestrus. On days when ewes were in oestrus (S+ sample donors) the S- sample was collected either from ewes that were on day 9 of their cycles, or from ewes that were the closest to this day (ranges 7 to 11; Tables 5, 6 and 7).



Experiment 5: Baseline of discriminatory ability.

The rams completed 5 days of olfactory discrimination trials using fresh oestrous and fresh non-oestrous ewe urine odours. The purpose of these trials was to re-establish baselines of discriminatory ability and ensure that rams were discriminating between odours prior to conducting experiment 6. The stimulus odours and donor ewes are shown in Table 5.

Experiment 6: Effect of blocking the NPC.

Rams C and D were fitted with bungs which completely occluded the NPCs and VN ducts as described. Rams A and B were untreated. All rams completed 5 days of experiments using fresh oestrous and non-oestrous ewe urine odours. The first trial was conducted on the same day that the bungs were fitted (4 days after experiment 5). The stimulus odours and ewe urine donors are shown in Table 6.

Experiment 7: Effect of  $\text{ZnSO}_4$  irrigation of both nostrils.

Ten days after experiment 6, Rams C and D were anaesthetised and their nostrils irrigated with 1%  $\text{ZnSO}_4$  and 4% Procaine solution as described. The NPC bungs were removed after the irrigation. The rams were allowed 3 days to recover from the procedure and then completed 5 trials using fresh oestrous and fresh non-oestrous urine. The stimulus odours used are shown in Table 7.



TABLE 5. Fresh oestrous and non-oestrous urine donors used in Experiment 5.  
Day of cycle for non-oestrous ewes in parentheses.

TRIAL	DATE	OESTROUS DONOR (S+)	NON-OESTROUS DONOR (S-)
1	28/1/89	180	181 (11)
2	30/1/89	197	198 (9)
3	4/2/89	181	180 (8)
4	8/2/89	198	197 (10)
5	9/2/89	198	197 (11)

TABLE 6. Fresh oestrous and non-oestrous urine donors used in Experiment 6.  
Day of cycle for non-oestrous ewes in parentheses.

TRIAL	DATE	OESTROUS DONOR (S+)	NON-OESTROUS DONOR (S-)
1	13/2/89	180	198 (7)
2	15/2/89	199	198 (9)
3	22/2/89	181	180 (10)
4	24/2/89	198	197 (9)
5	1/3/89	180	181 (8)

TABLE 7. Fresh oestrous and non-oestrous urine donors used in Experiment 7.  
Day of cycle for non-oestrous ewes in parentheses.

TRIAL	DATE	OESTROUS DONOR (S+)	NON-OESTROUS DONOR (S-)
1	13/3/89	197	199 (8)
2	15/3/89	198	199 (9)
3	17/3/89	180	181 (8)
4	29/3/89	181	199 (9)
5	31/3/89	197	199 (11)

### Rams ability to flehmen and detect aversive odours.

Immediately after each of the 5 trials in experiments 6 and 7, olfactory abilities were tested by:

- a) Investigating the rams ability to flehmen when fresh ewe urine was sprayed at the nose from a 5ml syringe.
- b) The odour aversion test of Poindron (1974).

### Control experiments.

Control experiments were conducted after experiment 7 was completed. Fresh oestrous urine odour was delivered to both sides of the crate simultaneously. If the rams were using confounding cues to complete the experiments they would have been able to select the switch associated with S+ preferentially, despite the absence of odour cues.

## RESULTS.

### Experiment 5.

All rams could discriminate between the odours of fresh oestrous and fresh non-oestrous ewe urine (figure 17) which was in agreement with the results of chapter 5. This was the case despite using urine from different donors and different oestrous cycles on each day. The error scores obtained were not significantly different to those obtained previously (Chapter 5). The Suffolk rams (A and D) had lower mean error scores (26 and 25) than the Cheviot rams (40 and 41). Analysis of the 2 sets of error scores (students t-test) showed no significant differences between the discriminatory abilities of rams here and in previous work using the

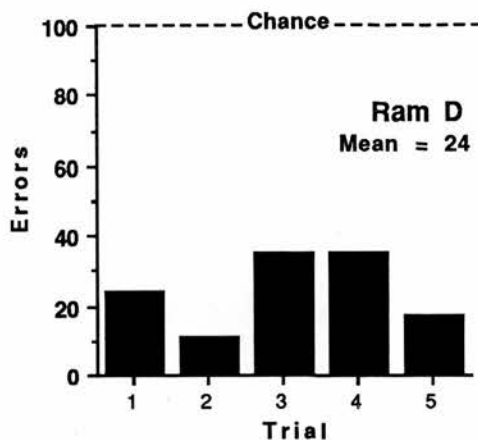
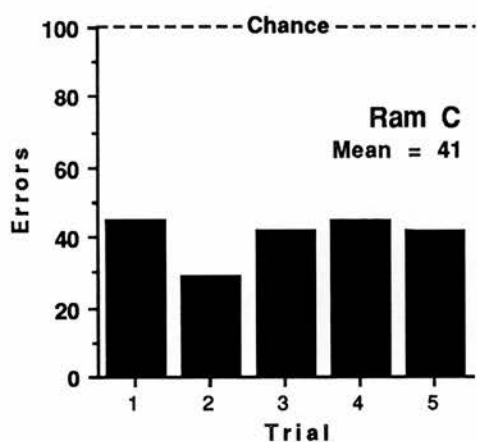
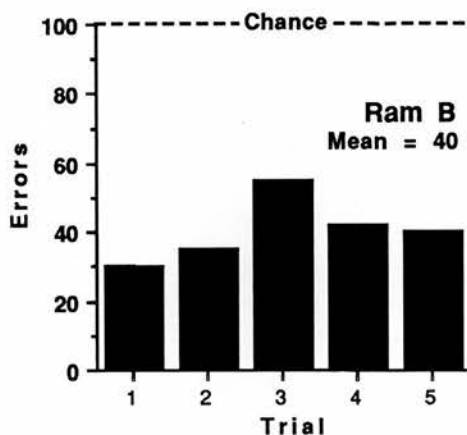
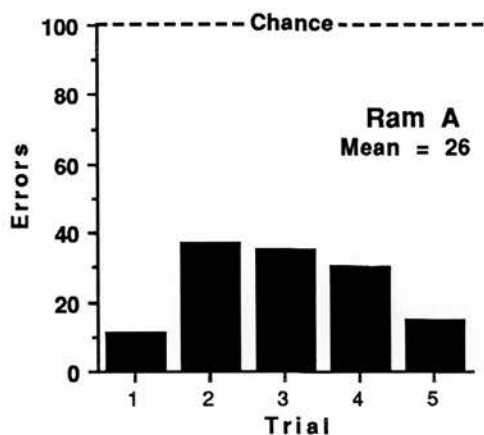


Figure 17. Error scores for four rams during five olfactory discrimination trials using fresh oestrous and non-oestrous ewe urine.

same type of stimulus odours.

#### Experiment 6.

Blocking the NPC, and the VN duct, had no effect on the rams ability to discriminate between fresh oestrous and non-oestrous urine odours. The overall mean error scores were higher in this experiment than in experiment 5, for each ram (figure 18) but there were no significant differences ( $P > 0.05$ ) between the mean error scores of any combination of 2 rams in this experiment, or between any ram's mean error score in experiment 5 and any that in experiment 6 (students T-test).

#### Experiment 7.

Conversely, the effect of intranasal  $ZnSO_4$  irrigation was marked (figure 19). The 2 untreated animals could discriminate between odours as expected but the 2 treated rams (C and D) showed profound deficits. The daily error scores of Ram C were above chance levels for all trials. The deficit outlasted the duration of the experiment ( $> 21$  days) even though the ram showed signs of improving olfactory acuity (decreasing error scores) over the 5 trials of experiment 7, it remained unable to discriminate by the final trial. Ram D scored above chance levels for the first 3 trials. The deficit had disappeared by the fourth trial (8 days postoperatively) and subsequent error scores returned to below chance levels.

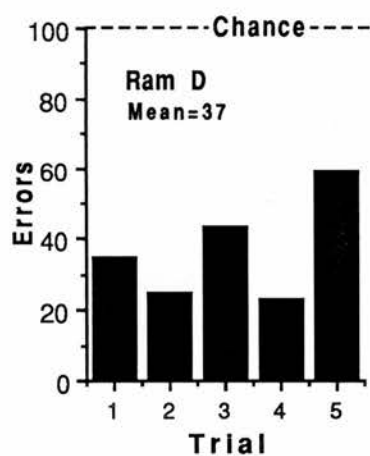
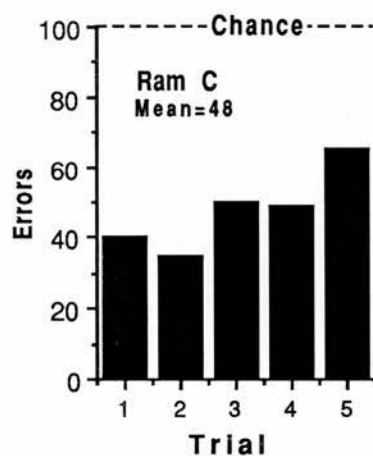
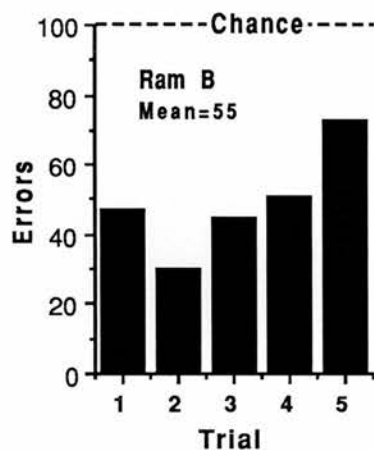
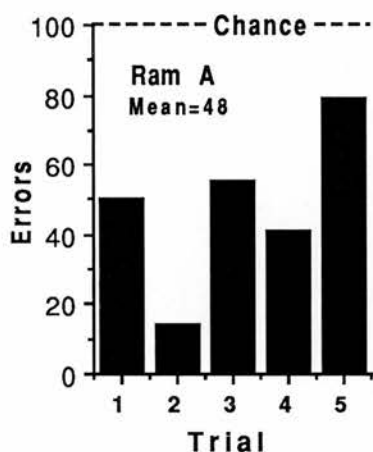


Figure 18. Error scores for four rams during five olfactory discrimination trials using fresh oestrous and non-oestrous ewe urine. Ram C and Ram D - nasopalatine canal occluded.



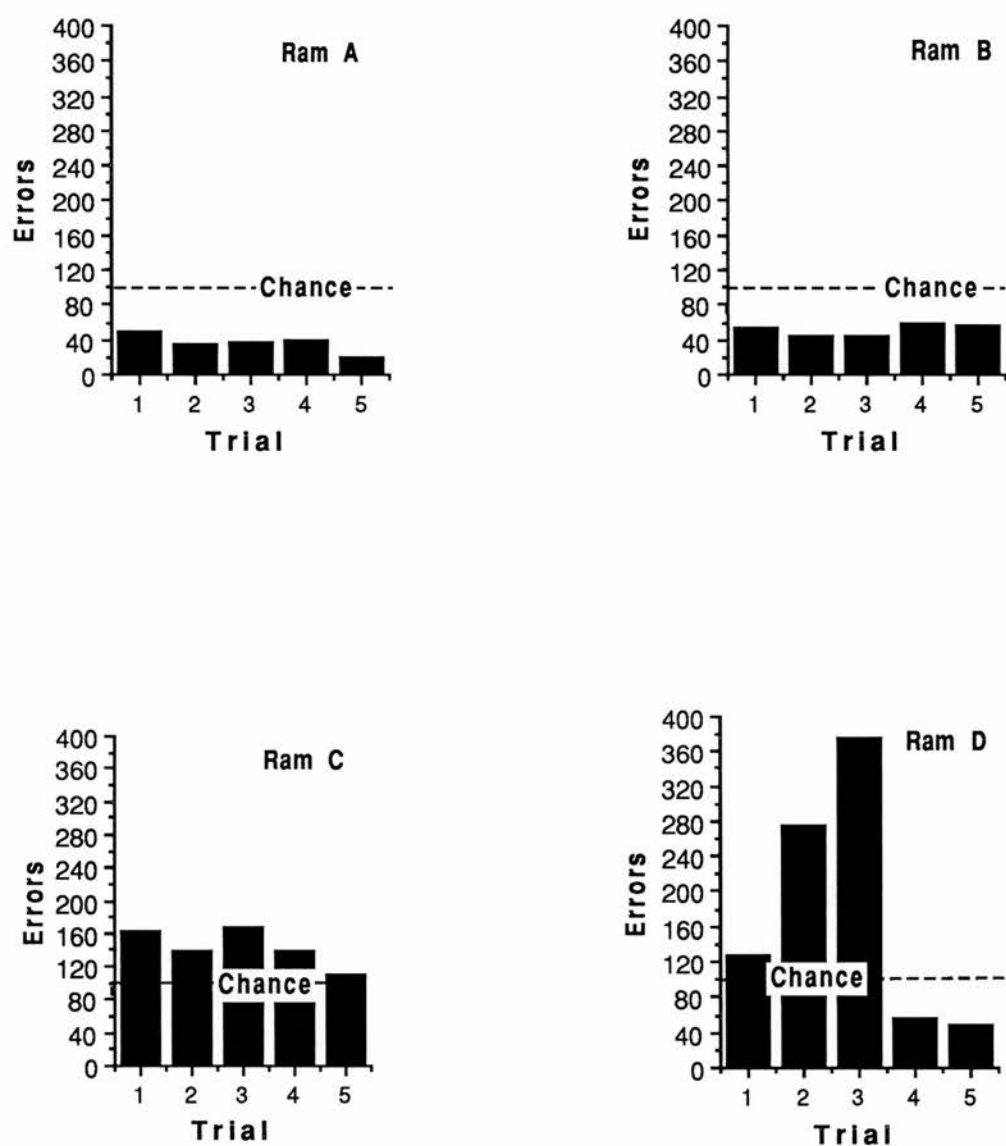


Figure 19. Error scores for four rams during five olfactory discrimination trials using fresh oestrous and non-oestrous ewe urine.  
Ram C and Ram D - Zinc sulphate nasal irrigation.

### Flehmen responses.

Intact rams and rams treated with NPC bungs flehmened in response to ewe urine sprayed at the nose at every presentation. The duration of the flehmen responses was not individually recorded but lasted beyond a minute in one case. Following treatment with  $\text{ZnSO}_4$  there was an individual variation in the flehmen response. Ram D did not flehmen when tested after the first 3 trials following  $\text{ZnSO}_4$  treatment but did so after every subsequent trial. Flehmen was not seen in Ram C after  $\text{ZnSO}_4$  irrigation.

### Aversive odours.

Intact rams and rams fitted with NPC bungs never ate from the contaminated bucket, indicating a normal ability to smell the aversive odour of dog faeces. Following  $\text{ZnSO}_4$  treatment, Ram C (who could not discriminate the urine odours or flehmen) ate from the contaminated bucket every day, indicating an inability to perceive the aversive odour of dog faeces. Ram D however (although unable to discriminate between urine odours, or flehmen, for the first 3 trials after nasal irrigation) refused to eat from the contaminated bucket after every trial. Control experiments were conducted using the same bucket without dog faeces odour, in which case the ram would eat normally.

## DISCUSSION.

These results provide the first information about the location of chemoreceptors mediating the discrimination between fresh oestrous and non-oestrous ewe urine odours by rams. Experiment 5 demonstrated the repeatability of previous results showing that rams could be trained to demonstrate their discrimination between these odours for a food reward. The levels of accuracy achieved here, in terms of the number of errors scored, were similar to previous results. The Suffolk rams again scored less errors (26 and 25) than the 2 Cheviots (40 and 41). These scores are higher than previously achieved though not significantly so ( $P > 0.05$ , students t-test). It was suggested previously that the superior performance of the Suffolks may be due to a superior technique (see page 100). This may be the case in these experiments. These results emphasise the reliability of the technique and the differing abilities of rams.

It is curious that olfactory deficits could not be induced using intranasal applications of local anaesthetic agents, especially as other workers report the success of the technique. The agents in this work were tested on oral mucosae of human subjects and successfully reduced the sensation of touch thereby demonstrating their activity. The methods of application which were employed failed to apply enough local anaesthetic agent to the olfactory epithelium to produce

an olfactory deficit.

Occluding the NPC and VN duct entrance had no effect on the ability of rams to discriminate between the two odours. The error scores of treated rams were not significantly different from the intact rams. These results demonstrate that the VNO is not necessary, in the adult ram, for discrimination between oestrous and non-oestrous urine odours. Neither does the ram require a patent NPC to achieve these discriminations. The VNO was therefore not the location of chemoreceptors required to mediate the discrimination of urine odours from ewes at different stages of the sexual cycle, in these experiments.

In contrast, after rams nasal cavities were irrigated with  $\text{ZnSO}_4$  solution a marked olfactory deficit was apparent. The respective durations of the deficits in rams C and D reflect the length of time that the irrigating fluid was left in the nose of each sheep. These results demonstrate that the chemoreceptors mediating the discrimination are almost certainly located intranasally. The irrigating fluid would have contacted the soft palate and pharyngeal tissues as well as intranasal surfaces, but the most likely cause of the behavioural deficit is damage to primary afferent olfactory neurones in the main olfactory epithelium. The decision to use reversible sensory deprivations precluded histological verification of lesions. It was

not possible to examine the damage caused to the intranasal sensory systems.

It is known however, that intranasal  $\text{ZnSO}_4$  causes a coagulative necrosis of the olfactory sensory epithelium (Smith 1938). The corresponding behavioural deficits are reversible (Alberts 1974; Murphy 1976). The histological events have not been documented in sheep, but have been for the rabbit (Mulvaney & Heist 1971) mouse (Matulionis 1975, 1976; Harding, Getchell & Margolis 1978; Burd & Margolis 1980) rat (Butler et al. 1984) and catfish (Cancalon 1982). It is clear from those studies that the effects of Zinc Sulphate can be highly variable depending on the concentrations of fluid used and the length of time the fluid is in contact with the tissues. Primary afferent olfactory neurones are continually being replaced in mammals about every 30 days (Takagi 1971; Monti Graziadei & Graziadei 1979;

Booth, Baldwin, Poynder, Bannister & Gower 1987) and this regenerative capacity may be a factor in the return of functional abilities following treatment. Previous work with sheep, using the same irrigation methods employed here, demonstrated behavioral deficits lasting from less than 8 hours to more than 21 days (Poindron 1974 and personal communication). The deficits in treated rams in these experiments (7 days and >21 days) are consistent with this and reflect the length of time the zinc sulphate was left in the nose of each ram prior to drainage. A



longer duration of treatment would be expected to increase the number of sensory cells destroyed. The evidence suggests that the chemoreceptors mediating the discrimination are located intranasally in the main olfactory epithelium.

The two other methods of assessing olfactory ability provided interesting results. All intact rams and rams with NPC bungs were able to flehmen in response to fresh ewe urine. The VNO is therefore not a necessary component of the sensory side of the flehmen response in adult rams. It is clear that adult rams deprived of a stimulus access route to the VNO can still flehmen. The character and durations of the flehmen responses did not appear to be different to those seen in intact rams although accurate quantification was not employed.

It is interesting that rams performed flehmen in response to urine spray but not to urine odours in the crate. The absence of flehmen in the crate may be because the odours were presented out of their normal context. Flehmen may only be used for more difficult discriminations involving urine from ewes at closer stages of the oestrous cycle than those used here. Another possibility is that urine volatiles play no part in initiation of flehmen and that this role is achieved entirely by less volatile, liquid phase chemicals. A further possibility is that it is the concentration of

stimulus and not the volatility of the particular urine stimulus which is important in provoking flehmen. If this is so the equipment used in this work may be deficient at providing sufficient concentrations of stimulus to initiate flehmen.

The ability to flehmen returned in Ram D at the same time as the ability to discriminate between urine odours in the crate. It is therefore probable that the same chemoreceptors mediate both olfactory behaviours and that they are olfactory chemoreceptors located in the main olfactory epithelium. Indeed, previous reports indicate that olfactory bulbectomy abolishes flehmen in rams (Lindsay 1965) and "virtually" (sic) abolishes it in goats (Ladewig et al. 1980).

All intact and NPC occluded rams refused to eat oats from the bucket contaminated with dog faeces odour. They could detect aversive odours and the VNO did not therefore mediate the perception of this aversive odour. Following nasal irrigation Ram C ate from the contaminated bucket on every occasion. Ram C was therefore "anosmic" according to all 3 methods of testing. Ram D refused to eat from the contaminated bucket after every trial, despite not being able to discriminate urine odours or perform flehmen in the first 3 trials. The  $\text{ZnSO}_4$  selectively deprived the ram of the ability to discriminate urine odours and flehmen but spared the ability to perceive strongly aversive odours. It may be that different chemoreceptors mediate

the 2 different types of odour stimuli (e.g. an undamaged trigeminal input may have mediated the aversive stimulus) or alternatively the strongly aversive odour may have been present in concentrations which were easier to detect. Whilst Ram C could be described as "anosmic" in all 3 tests used after nasal irrigation, Ram D could only be described as "anosmic " in terms of not being able to discriminate urine odours or flehmen to ewe urine. According to Poindron's (1974) test Ram D was able to smell the aversive odour of dog faeces all the time.

There are no previous reports of the effects of VNO deprivation in rams. Techniques employing VN nerve section and cauterisation of the VNO have shown that the release of luteinising hormone (LH) in ewes exposed to ram odour is not mediated by the VNO (Cohen-Tannoudji, Lavenet, Locatelli, Tillet & Signoret 1989). Olfactory ablation in ewes (destroying the main and accessory olfactory systems) does prevent the ewes LH response to rams (Cohen-Tannoudji et al. 1989) indicating that the main olfactory system mediates the endocrine response.

The results of this work support the hypothesis that in sexually experienced sheep, the main olfactory system mediates the perception of conspecific odours important in reproduction. This appears to be different to rodents where the VNO has an important chemosensory role (Reynolds & Keverne 1979; Bellringer et al. 1980;

Lomas & Keverne 1982; Wysocki, Katz & Bernhard 1983; Coquelin et al. 1984)

Recent studies indicate that the VNO may be important in mediating chemical cues in sexually inexperienced animals, and that the main olfactory system becomes important in the perception of conditioned odour cues so that the VNO becomes less important in later life (Keverne et al. 1986). Future work should determine if the VNO has such a role in sexually inexperienced ram lambs.



### GENERAL DISCUSSION.

It has been demonstrated in this work that rams can detect oestrous ewes by the odour of their urine. This is also the case with males of some other species (Brown 1979), including dogs (Doty & Dunbar (1974), rats (Lydell & Doty 1972), gerbils (Pettijohn 1974) and Asian elephants (Rasmussen, Schmidt, Henneous, Groves & Doyle-Daves 1982) but not mice (Hayashi & Kimura 1974), guinea pigs (Beauchamp et al. 1972) or horses (Stahlbaum & Houpt 1989). It is not clear at what stage of the ovine oestrous cycle the odour of non-oestrous urine changes to that of oestrous urine, but this could be determined by conducting olfactory discrimination experiments using non-oestrous urine and urine from days of the cycle progressively approaching oestrus. If such a change occurred at "pro-oestrus" then certain aspects of ovine courtship behaviour become clearer. It would be advantageous for non-oestrous ewes to urinate frequently to the approaching ram - either to indicate the non-receptive state and reduce harassment, or to indicate their impending change of sexual state and increase the likelihood of a ram being present at this time. It would also be advantageous for rams to investigate ewe urine to determine a ewes impending sexual receptivity. The "standing reaction" of an oestrous ewe is the most important indicator of oestrus to the ram. The ram can obtain odorous information at this time by close genital investigation and urination by the ewe is unnecessary.



The experiments described here have established that in adult rams the VNO is not required for discrimination between volatiles in oestrous and non-oestrous ewe urine. However, no attempt has been made to determine the role of the VNO in discriminations between less volatile urine components, or "odours in solution", which rams have available to them under field conditions. To preclude this possibility and eliminate the VNO as a chemosensory organ with a role in the detection of oestrus, it is necessary to compare the oestrus detection rates of intact and VNO occluded rams in field mating trials. The VNO may be important for aspiration of a liquid column of urine (Bland & Cottrell 1989) - see page 129.

It was expected that the main olfactory epithelium and not the VNO is a necessary sensory component of the flehmen response. Rams do not flehmen to voided ewe urine from a distance but approach them and perform close nasal investigations prior to flehmen. Olfactory deficits would prevent both the identification and localisation of stimuli.

Recent work has demonstrated a mechanism for VNO filling in the anaesthetised ram (Bland & Cottrell 1989). The intraluminal pressure of the VNO in the ram is generally just above atmospheric pressure, but occasional spontaneous falls to below atmospheric pressure suggest a mechanism for regular sampling of

chemosignals by the VNO. In addition, sympathetic stimulation by either administration of adrenaline tartrate, or by electrical stimulation of the ipsilateral cervical sympathetic trunk, produces large falls in intraluminal pressure ( up to -25mmHg). By placing a catheter containing 0.9% saline in the buccal end of the nasopalatine canal it has been shown that the pressure fall in the VNO causes fluid to be drawn into the organ. The fall in pressure is quite rapid but the recovery phase is slower, lasting for up to 2 minutes in one case. It is interesting that the durations of the falls and subsequent rises in pressure are very similiar to recorded durations of flehmen in the ram.

#### The future.

It remains to be seen whether such a filling mechanism is activated during flehmen. It will be necessary to record intraluminal VNO pressures from conscious rams to determine if the VNO fills when the ram flehmens. The purpose of the bizarre postural act may be more difficult to determine.

There remains no direct evidence of a role for the VNO in ruminants, however the method of occluding stimulus access, described here, provides an easy and reversible means of determining if the VNO is necessary for particular behaviours. The technique could be beneficially employed to determine if the VNO is important in mediating primary unconditioned sexual cues in sexually inexperienced ram lambs, as has been

suggested for other species (Keverne et al. 1986).

The ewe has a VNO which is indistinguishable from that of the ram. Flehmen in the ewe is most commonly reported around parturition. The technique of VNO occlusion could be employed to determine if the VNO has a role in the selective bonding of ewes and lambs mediated by chemosignals.

The remaining central problem is the identification of the chemical difference between ewe urine volatiles at oestrus and non-oestrus. The methods of olfactory discrimination in this work provide a bioassay which could be combined with a suitable analytical technique to determine the identity and nature of ovine "oestrous indicating pheromones".

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## APPENDIX A.

Computer programme developed to control the  
mechanisms of the operant conditioning crate.

```
10 CLS
30 PROCREADFILE
40 STARTN=N
50 PROCMENU
60 IF SERIAL NO=0 AND N<>STARTN THEN PROCWRITEFILE
70 IF SERIAL NO=0 THEN END
80 IF SERIAL NO<=N THEN PROCEXPERIMENT
90 IF SERIAL NO=N+1 THEN PROCNEWSERIES
100 IF SERIAL NO=N+2 THEN PROCPAGE
110 IF SERIAL NO=N+3 THEN PROCMENU
120 GOTO 50
130 REM
140 REM
150 DEF PROCINIT
160 ?&FCC3=225
170 ?&FCC2=15
180 ?&FCC0=0
190 ?&FCC1=192
200 ENDPROC
210 REM
220 REM
230 DEF PROCREADFILE
240 A=OPENIN"SERIES"
250 INPUT#A,N
260 NS=N+5
270 DIM SERIES$(NS,50)
280 FOR I=1TON
290 FOR J=1TO50
300 INPUT#A, SERIES$(I,J)
310 NEXT
320 NEXT
330 CLOSE #A
340 ENDPROC
350 REM
360 REM
370 DEF PROCMENU
380 CLS
390 PRINT"SERIES NUMBER          FIRST 10 MEMBERS"
400 PRINT
410 FOR I=1TO7
420 PRINT I;".....";
430 FOR J=1TO10
440 PRINT;SERIES$(I,J);" ";
450 NEXT
460 PRINT
470 NEXT
480 PRINT
```

```

490 K=N+1
500 PRINT K;".....ENTER NEW SERIES"
510 PRINT
520 Z=N+2
530 PRINT Z;".....FOR MORE SERIES"
540 PRINT
550 PRINT "          0=QUIT"
560 PRINT
570 PRINT
580 INPUT"TYPE IN REQUIRED NUMBER >" SERIAL NO
590 ENDPROC
600 REM
610 DEF PROCPAGE
620 CLS
630 FOR I=8TON
640 PRINT I;".....";
650 FOR J=1TO10
660 PRINT;SERIES$(I,J);" ";
670 NEXT
680 PRINT
690 NEXT
700 PRINT
710 K=N+1
720 PRINT K;".....ENTER NEW SERIES"
730 PRINT
740 Q=N+3
750 PRINT Q;".....TO PREVIOUS PAGE"
760 PRINT
770 PRINT "          0=QUIT"
780 PRINT
790 PRINT
800 INPUT"TYPE IN REQUIRED NUMBER >"SERIAL NO
810 GOTO 60
820 ENDPROC
830 REM
840 DEF PROCEXPERIMENT
850 INPUT"TO COMMENCE TRIAL TYPE START >"REPLY$
860 IF REPLY$="START" THEN GOTO 870 ELSE GOTO850
870 FOR P=1TO50
880 IF SERIES$ (SERIAL NO,P)="L" THEN PROCLEFT
890 IF SERIES$ (SERIAL NO,P)="R" THEN PROCRIGHT
900 IF SERIES$ (SERIAL NO,P)<>"R" AND SERIES$
(SERIAL NO,P)<>"L"THEN PROCALARM
910 NEXT P
920 ?&FCC0=0:REM ALL RELAYS OFF
930 CLS
940 PRINT "END OF TRIAL PLEASE REMOVE SHEEP"
950 SOUND 1,-15,52,10
960 PRINT "PRESS ANY KEY TO CONTINUE"
970 KEY$=GET$
980 ENDPROC
990 REM
1000 REM
1010 DEF PROCALARM
1020 SOUND 1,-15,148,60

```

```

1030 PRINT"SERIES CONTAINS AN ERROR WHICH IS NOT AN
L OR AN R."
1040 FOR I=1TO50
1050 PRINT SERIES$(SERIAL NO,I);
1060 NEXT

1070 PRINT
1080 PRINT "ENTRY NUMBER ";P" IN THE SERIES READS ";
SERIES$(SERIAL NO,P);" PLEASE CORRECT>";
1090 INPUT , SERIES$(SERIAL NO,P)
1100 IF SERIES$(SERIAL NO,P)<>"L" AND SERIES$(SERIAL
NO,P)<>"R" THEN GOTO1080 ELSE PROCMENU
1110 REM
1120 REM
1130 DEF PROCWRITEFILE
1140 CLS
1150 B=OPENOUT "SERIES 2"
1160 PRINT #B,N
1170 FOR I=1TON
1180 FOR J=1TO50
1190 PRINT#B,SERIES$(I,J)
1200 NEXT
1210 NEXT
1220 CLOSE#B
1230 *DELETE"SERIES"
1240 *RENAME SERIES 2 SERIES
1250 PRINT"REMOVE DISC AND TURN OFF COMPUTER AND
INTERFACE"
1260 .ENDPROC
1270 REM
1280 CLS
1290 REM
1300 DEF PROCNEWSERIES
1310 N=N+1
1320 PRINT
1330 PRINT"ENTER NEW GELLERMAN SERIES OF LEFT AND
RIGHTS USING THE LETTERS L AND R."
1340 I=1
1350 REPEAT
1360 KEY$=GET$
1370 PRINT;KEY$;" ";
1380 IF KEY$="L" OR KEY$="R" THEN SERIES$(N,I)=KEY$
ELSE PROCBADKEY
1390 I=I+1
1400 UNTIL I=51
1410 CLS
1420 PRINT"NEW SERIES ENTERED IS:"
1430 PRINT
1440 FOR I=1TO50
1450 PRINT;SERIES$(N,I);
1460 NEXT
1470 PRINT
1480 PRINT
1490 PRINT"PLEASE CHECK THIS IS CORRECT i.e. CORRECT
ENTRIES AND CORRECT ORDER"

```

```

1500 PRINT "IF CORRECT TYPE OK"
1510 INPUT "IF INCORRECT TYPE REDO AND RE-
ENTER>"ANSW$
1520 IF ANSW$="OK" THEN ENDPROC
1530 IF ANSW$="REDO" THEN 1320 ELSE GOTO 1420
1540 ENDPROC
1550 DEF PROCBADKEY
1560 SOUND 1,-15,100,10
1570 PRINT
1580 PRINT "ERROR - TYPE C TO CANCEL LAST ENTRY";
1590 REPEAT:ANS$=GET$:UNTIL ANS$="C":PRINT;"C"
1600 I=I-1
1610 FOR J=1TOI:PRINT;SERIES$(N,J);" ";:NEXT
1620 ENDPROC
1630 REM
1640 REM
1650 DEF PROCLEFT:REM TO BE DONE WHEN L IN SERIES
1660 PRINT ?&FCC0
1670 IF ?&FCC0=240 OR ?&FCC0=254 THEN ?&FCC0=249
ELSE ?&FCC0=240
1680 PRINT ?&FCC0
1690 PRINT "WAITING FOR SHEEP TO FINISH LEFT ";P
1700 X%=?&FCC0: IF X%>128 THEN GOTO 1700
1710 X%=?&FCC0: IF X%<128 THEN GOTO 1710
1720 FOR S=1TO500
1730 NEXT S
1740 ENDPROC
1750 REM
1760 REM
1770 DEF PROCRIGHT:TO BE DONE WHEN R IN SERIES
1780 PRINT ?&FCC0
1790 IF ?&FCC0=249 OR ?&FCC0=247 THEN ?&FCC0=254
ELSE ?&FCC0=247
1800 PRINT?&FCC0
1810 PRINT "WAITING FOR SHEEP TO FINISH RIGHT ";P
1820 X%=?&FCC0: IF X%>128 THEN GOTO 1820
1830 X%=?&FCC0: IF X%<128 THEN GOTO 1830
1840 FOR S=1TO500
1850 NEXT S
1860 ENDPROC

```